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Two Parts
Part 1

THE
BIOLOGICAL BULLETIN

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SEXUAL REPRODUCTION WITHOUT CROSS-COPULATION IN THE FRESH-WATER TRICLAD TURBELLARIAN, CURTISIA FOREMANII

JOHN MAXWELL ANDERSON

Arnold Biological Laboratory, Brown University, Providence 12, Rhode Island

The occurrence of self-fertilization among fresh-water triclad Turbellaria has been repeatedly denied on the basis of studies carried out with several species (Vandel, 1921; Gelei, 1924; Goetsch, 1925). The availability of vigorous mass cultures of an American species (*Curtisia foremanii*) not previously investigated in this respect presented an opportunity to determine for this species whether cross-copulation is necessary for the production of cocoons containing fertile eggs. The purpose of this paper is to report on preliminary experiments designed to answer this and related questions. The results of these experiments show clearly that cross-fertilization is not at all essential in the sexual reproduction of this species, which does not reproduce asexually.

Thanks are due Dr. C. William Nixon for supplying the original specimens from which all our worms have been cultured, and for preparing the strains used for the observations. It is a pleasure to acknowledge the painstaking technical assistance of Dorothy T. Clarke and Alice E. Watjen in maintaining the isolated worms and in keeping the voluminous records required.

MATERIALS AND METHODS

The worms used in these observations were descendants of several specimens of *Curtisia foremanii* collected in September, 1949, from a pond in Lincoln Woods State Park, near Providence, and maintained in the laboratory since that time. For purposes not directly concerned with the present investigation, it was desirable to produce two different strains of worms, each as genetically homogeneous as possible. Toward this end, two strains (A and B) were prepared as follows. For each strain, one mature worm, selected from a mass culture, was cut transversely into two pieces which were allowed to regenerate. Each of these regenerates was then cut into two pieces as before. The four worms produced by regeneration of these pieces were maintained in a finger-bowl and, together with their subsequent offspring, constituted the original population from which cocoons were obtained for experimentation. A diagrammatic presentation of this procedure appears in Figure 1.

Three cocoons were selected from Strain A and four from Strain B. Each cocoon was assigned a number and isolated. Upon hatching, the juveniles from each cocoon were isolated and kept separately in 50 mm. stender dishes. This isolation was always carried out on the day of hatching, or on the first day following. Litter-mate worms, hatching from the same cocoon, were assigned numbers indicating the cocoon from which they emerged. Nothing is known of the parentage of worms in this generation, except that the culture producing them came originally from one or another of two single worms as described above. Worms hatching from these isolated cocoons are referred to as the P_1 generation.

FIGURE I

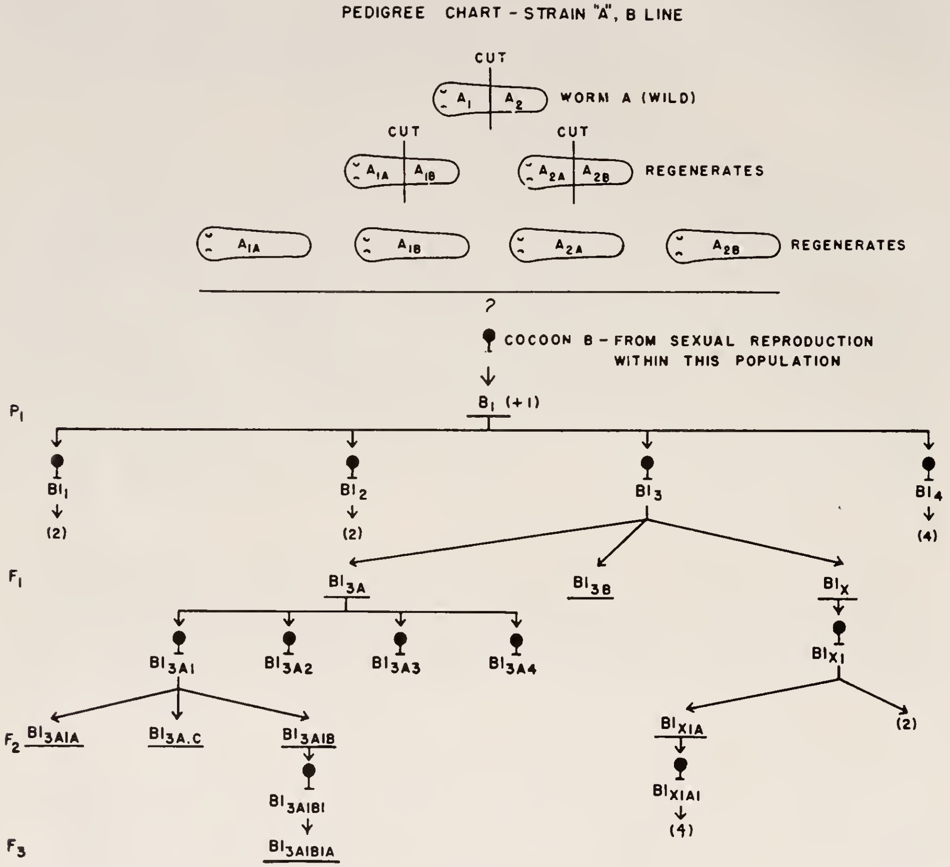
PEDIGREE CHART - STRAIN "A", B LINE
CUT
A1
A2
WORM A (WILD)
CUT
CUT
A1A
A1B
A2A
A2B
REGENERATES
A1A
A1B
A2A
A2B
REGENERATES
?
COCOON B - FROM SEXUAL REPRODUCTION WITHIN THIS POPULATION
B1 (+1)
P1
BI1
(2)
BI2
(2)
BI3
BI4
(4)
F1
BI3A
BI3B
BIX
BIX1
BI3A1
BI3A2
BI3A3
BI3A4
F2
BI3A1A
BI3A.C
BI3A1B
BI3A1B1
BIX1A
BIX1A1
(4)
(2)
F3
BI3A1B1A

Figure 1.

All young worms thus isolated were kept in water from a greenhouse pool and fed to repletion with mouse liver twice a week. Dishes were carefully cleaned and the water changed after each feeding. The dishes were stacked in trays on a shelf away from direct sunlight, at room temperature. As the P_1 worms neared maturity, they were observed daily, and the date of deposition of the first cocoon recorded for each individual. Each cocoon, and each juvenile hatching from it and subsequently isolated, was numbered for identification. The numbering scheme (see Fig. 1) is designed to show the entire pedigree of the cocoon or worm.

After each cocoon hatched, the young worms were isolated, fed, and observed as described above. As the population increased, it became necessary, owing to limitations of time and space, to discontinue many of the lines and to maintain only a selected few.

It should be emphasized that young worms were isolated from one another as soon as possible after their emergence from the cocoon. Usually this was done on the day of hatching; occasionally, isolation was deferred until the third or fifth day following hatching. Serial sections of one- and three-day-old worms were searched for signs of precocious development of the genital systems. Since no indication of such unusually early maturation was detected, it is considered extremely improbable that cross-copulation could have occurred between these young worms before isolation.

These observations were begun in January, 1951; in July, a steady increase in mortality in the cultures and cessation of reproductive activities indicated that midsummer laboratory temperatures were above the optimal level. Therefore, the cultures were removed to a cool basement room, and within about three weeks the worms began to deposit cocoons again at an accelerated rate. Temperatures in the neighborhood of 25° C., common in the laboratory in summer, evidently inhibit the entire reproductive process. Previous experiments (Anderson, unpublished) have established the fact that cocoons of Curtisia will not hatch when incubated at a constant temperature of 25° C.

OBSERVATIONS

Table I presents a compilation of the breeding record, through mid-September, 1951, of the 15 worms of the P_1 generation. Worms with the same capital-letter designation are litter-mates, *i.e.*, hatched from the same cocoon. It will be noted that with the exception of A2 and D1, all worms of this generation, each kept in strict isolation almost from the day of hatching, have produced at least two cocoons, and that most have deposited more than two.

It is also clear from this table that cross-copulation is not necessary for the production of fertile eggs. P_1 worms have produced a total of 48 cocoons; 3 were lost, and 13 have not attained hatching age. Of the remaining 32 cocoons, only 7 have proved infertile. The fertility rate for P_1 cocoons is thus approximately 78%.

The number of young (F_1) worms produced by each cocoon is variable. In the 15 hatched cocoons from individuals of Strain A, the number of young varies between 1 and 6, with a mean of 2.5 juveniles per cocoon. For the 10 hatched cocoons from P_1 worms of Strain B, the range is from 1 to 4, and the mean is also approximately 2.5 young per cocoon.

Table I shows also the number of days elapsed between date of hatching and date of deposition of the first cocoon for each P_1 individual. No attempt was made to maintain the cultures under conditions of controlled temperature, so that variations in the length of this pre-reproductive period may be due, in part, to temperature variations. However, all P_1 individuals hatched within 3 days in January, and all were exposed to the same varying temperatures.

Large differences are apparent in the pre-reproductive periods, even those of litter-mate worms. For example, compare worm C1 with C2, and F1 with its two litter-mates, F2 and F3. It should be noted that A2 deposited its first cocoon on 30 August, while all other P_1 individuals had produced cocoons prior to the onset of the heat-induced depression in July. Therefore, the extremely long pre-reproductive period of A2 is probably abnormal. Leaving A2 out of account, the mean pre-reproductive period for all other P_1 worms is about 79 days; in Strain A, this period is 20 days shorter on the average (69 days) than in Strain B (89 days). Because of the comparatively small number of individuals and the absence of temperature control, these figures lack statistical validity. However, they may be useful in indicating the approximate length of time required for *Curtisia foremanii* individuals to reach sexual maturity under these conditions, and in demonstrating the considerable variation in the duration of the pre-reproductive period between different individuals.

TABLE I

Breeding record of isolated P_1 worms

Worm #	Date hatched	Date of 1st cocoon	Days to maturity	Total cocoons	Total young, (F_1)
Str. A					
B1	17 Jan.	26 Feb.	40	4(4f)	9
B2	17 Jan.	25 Mar.	67	3(1f, 2u)	3
C1	17 Jan.	9 May	111	3(2f, 1L)	4
C2	17 Jan.	27 Mar.	69	3(3f)	14
E1	19 Jan.	12 Mar.	53	5(2f, 3i)	5
E2	19 Jan.	2 Apr.	72	3(3f)	3
Str. B					
A1	16 Jan.	9 May	115	5(1f, 1L, 3u)	2
A2	16 Jan.	30 Aug.	(226)*	1(1u)	—
D1	17 Jan.	—	—	—	—
D2	17 Jan.	2 Apr.	74	5(3f, 1i, 1u)	7
F1	19 Jan.	21 June	151	2(1L, 1u)	—
F2	19 Jan.	7 Apr.	78	3 (1i, 2u)	—
F3	19 Jan.	7 Apr.	78	3(1f, 1u, 1i)	3
G1	19 Jan.	25 Mar.	65	4(2f, 1i, 1u)	6
G2	19 Jan.	21 Mar.	61	4(3f, 1u)	6

Note: f, fertile; i, infertile; u, unhatched; L, lost.
* Period abnormally lengthened by summer inhibition.

Table II presents similar data for isolated single worms of the F_1 generation. None of the 16 surviving worms of Strain A in this series has failed to produce cocoons, while two individuals of Strain B have failed to do so. The two sterile worms (D2–2A and D2–2B) are litter-mate offspring of P_1 worm D2, which was a litter-mate of the only sterile worm of the P_1 generation (D1). The 21 surviving fertile worms of this entire group of F_1 individuals have deposited a total of 41 cocoons. Nine of these have not attained hatching age; the fertility rate for the remaining 30 cocoons is about 93%, since two were infertile. A total of 91 juveniles emerged from the 28 fertile cocoons, giving a mean of slightly over three young per cocoon.

Only those worms which produced their first cocoons before 1 July should be considered as showing a normal pre-reproductive period. Ten Strain A worms and all of the Strain B worms in this group have abnormally prolonged prematurity periods. The mean length of the period in the remaining 6 Strain A individuals is 69 days, *i.e.,* the same as that encountered in the P_1 worms of Strain A. However, in the P_1 worms the range of variation is 40 to 111 days, while in the F_1 worms it extends from 61 to 86 days.

TABLE II

Breeding record of isolated F_1 worms

Worm #	Date hatched	Date of 1st cocoon	Days to maturity	Total cocoons	Total young (F_2)
Str. A:					
B1–1A	9 Mar.	7 May	61	2 (2f)	6
B1–3A	7 Apr.	11 June	65	4 (3f, 1u)	11
B1–3B	7 Apr.	30 Aug.	145*	1 (1u)	—
B1x =					
B1–3C	7 Apr.	11 June	65	1 (1f)	3
B1–4A	22 May	30 Aug.	100*	1 (1f)	4
B1–4B	22 May	21 Aug.	91*	1 (1f)	1
B1–4C	22 May	30 Aug.	100*	1 (1f)	1
B1–4D	22 May	30 Aug.	100*	1 (1f)	3
C1–1A	18 May	30 Aug.	104*	2 (2f)	6
C1–2A	16 June	(died without issue 20 July)			
C1–2B	16 June	(died without issue 1 July)			
C2–1x	6 Apr.	9 June	64	2 (2f)	5
C2–1A	6 Apr.	20 Aug.	136*	2 (2f)	7
C2–2A	5 May	20 Aug.	107*	2 (2f)	10
E1–1A	26 Mar.	5 June	71	2 (2f)	3
E1–1B	26 Mar.	20 June	86	4 (2f, 2i)	4
E1–2A	25 May	18 Aug.	85*	2 (2u)	—
E2–2A	5 May	30 Aug.	117*	2 (1f, 1u)	4
Str. B:					
A1–1A	26 May	18 Aug.	84*	3 (1f, 2u)	3
D2–1A	16 Apr.	30 Aug.	136*	1 (1u)	—
D2–2A	25 May	—	—	—	—
D2–2B	25 May	—	—	—	—
F3–1A	25 Apr.	21 Aug.	118*	1 (1f)	4
G2–1A	2 Apr.	18 Aug.	138*	4 (3f, 1u)	8
G2–1B	2 Apr.	20 Aug.	140*	2 (2f)	8

Note: f, fertile; i, infertile; u, unhatched.
* Pre-reproductive period abnormally lengthened by summer inhibition.

Fifteen F_2 worms have been maintained in isolation since shortly after hatching. Eleven of these are more than 2½ months old and may be considered old enough to reproduce. Of these, 6 have produced a total of 12 cocoons; the other 5 remain without issue. All cocoons have hatched; they produced a total of 47 young, for an average of almost four young per cocoon, with a range of two to five. None of the F_3 worms has yet attained sexual maturity.

In summary, 63 individuals of all generations (P_1, F_1, F_2, and F_3) have been maintained in isolation since shortly after hatching. Ten of these are too young to reproduce; 8 have deposited no cocoons although apparently old enough; 41 have produced at least one cocoon each. Altogether, 101 cocoons have been obtained from the 41 fertile isolated worms in three generations (P_1, F_1, F_2); 3 were lost, 9 were infertile, 22 remain unhatched, and 67 have produced young worms. Thus, from three generations of isolated worms, the overall percentage of fertility for cocoons of known condition, *i.e.*, disregarding lost and unhatched cocoons, is approximately 88%.

Figure 1 is a pedigree chart for parts of the B line, showing also details of preparation of Strain A. These worms and cocoons are descendants of one of the two juveniles which hatched from Cocoon B on 17 January 1951. All the worms shown were isolated from their litter-mates on or before the third day after hatching. Their offspring, therefore, have been produced without any opportunity for the occurrence of cross-copulation. Only the P_1 worms could have resulted from cross-fertilization involving two individuals.

Preliminary experiments have been begun to compare the reproductive performance of isolated worms with that of litter-mate worms maintained in groups. For example, one cocoon deposited by F_1 worm B1–1A hatched, releasing four litter-mate juveniles. One of these was isolated, the other three kept together. On the 97th day following hatching, the isolated worm deposited its first cocoon. On the same day, three cocoons appeared in the culture containing the three litter-mate worms. One of these three worms deposited an additional cocoon on the 109th day. Two juveniles emerged from the cocoon deposited by the isolated worm; only one of the four cocoons in the mass culture has hatched, releasing two juveniles. These experiments must be continued and expanded before any conclusions can be drawn regarding the comparative fertility of worms maintained with and without the opportunity for cross-copulation.

Several individuals with marked abnormalities have been obtained in these studies, among them those designated "x" in Table II. It was hoped that these individuals could be used in genetic studies; however, all of their offspring have been perfectly normal. No persistent, transmissible conspicuous peculiarities have become established in the cultures.

Discussion

As reported by Sekera (1906), self-fertilization is widespread among Rhabdocoela; Wilhelmi (1909) reported sexual reproduction in a single isolated individual of *Procerodes lobata* (Tricladida, Maricola). However, the present studies appear to be the first which have successfully demonstrated the possibility of autofecundation in fresh-water triclad Turbellaria. Vandel (1921, p. 436) was unable to obtain cocoons from isolated *Polycelis cornuta* individuals kept for one year; these worms were sexually mature but did not deposit cocoons in isolation. Placed together in a culture after one year, they still did not produce cocoons.

Gelei (1924) was unsuccessful in an attempt to demonstrate self-fertilization in isolated *Dendrocoelum lacteum* individuals. All cocoons deposited by 40 worms of this species failed to hatch. Gelei concluded that spermatozoa produced by one individual fail to become active unless transferred to another individual. Similar results were obtained by Goetsch (1925) from experiments with *Planaria lugubris,*[1] in which 31 infertile cocoons were deposited by 10 isolated individuals. After 10 months in isolation, two worms placed together proceeded to copulate and deposited fertile cocoons. Goetsch called attention to the fact that the production of young by isolated individuals would not constitute conclusive evidence for self-fertilization but would indicate equally the possibility of parthenogenesis. He concluded from his experiments that neither self-fertilization nor parthenogenesis occurs in fresh-water triclads.

---

[1] Hyman, in a personal communication, states that this species belongs in the genus Dugesia. The *Planaria lugubris* used in this country by Morgan, Stevens, and others for studies on regeneration is not the *Dugesia* (= *Planaria*) *lugubris* used by Goetsch, but *Curtisia foremanii.*

The latest authoritative statement concerning reproduction in fresh-water triclads appears in Hyman (1951, p. 155): "The fresh-water triclads are of course hermaphroditic but cross-copulation is necessary for fertilizing the eggs. Contrary to statements often found in zoological books, the triclads do not fertilize themselves and capsules laid by isolated individuals will not hatch as the eggs therein have not been fertilized. The construction of the reproductive system is such that self-fertilization is anatomically possible but the sperm do not leave the spermiducal vesicles except during an act of copulation and therefore never get into the ovovitelline ducts of the same individual." All previous experimental evidence completely justifies this statement. However, the observations outlined above on *Curtisia foremanii* demonstrate that this analysis of the situation is no longer valid.

There do appear to be differences in the readiness with which different individuals will produce fertile cocoons in isolation. The overall percentage of infertility in 41 mature worms of three generations (P_1, F_1, F_2) in these experiments is about 19%, indicating that in this species the number of worms apparently incapable of sexual reproduction under conditions of isolation is comparatively small. Without direct evidence for comparison, it nevertheless seems likely that the overall fertility rate of 88% in 101 cocoons is not significantly less than one might expect in a similar number of cocoons selected at random from a mass culture.

Experiments which were designed to investigate strain differences in productivity were impaired by the occurrence of the summer reproductive depression. However, it is clear that P_1 worms in Strain A reached maturity earlier than those in Strain B and produced a greater number of fertile cocoons (15:10) and more offspring (38:24). The remainder of the breeding record cannot be used for strain comparisons as Strain A, breeding earlier, was less affected by the summer depression that Strain B.

There is some evidence that inability to reproduce in isolation may be conditioned by genetic factors, as indicated by the comparatively high incidence of infertility in worms of the D line. Investigations on the performance of this line will be continued in an attempt to establish this point.

It should be stated that no evidence can be brought to bear from these studies on the question of parthenogenesis. As neither self-fertilization nor parthenogenesis has been previously described in fresh-water triclads, either possibility remains. These studies have shown only that cross-copulation, involving two individuals, is not a necessity in the reproduction of this species. "Self-copulation," described in Rhabdocoels and maricolous triclads, in which after appropriate folding of the posterior portion of the body, sperm may be injected directly into the seminal bursa of the same individual, has not been observed in Curtisia.

Further experiments are desirable to determine: whether large differences in reproductive potential exist between strains; whether reproductive potential declines after many generations of reproduction without the interchange of spermatozoa between individuals; whether (assuming parthenogenesis does not occur) self-mating, a most intensive form of inbreeding, will increase the frequency of abnormalities in the population; whether reproduction is more rapid and successful in a mass culture, where cross-copulation is possible, than among a similar number of isolated worms.

With regard to this last point, in almost two years' experience in maintaining mass cultures of Curtisia in this laboratory, none of the personnel involved has ever observed two worms in copulation, although the phenomenon has been continually sought in the cultures. One is tempted, therefore, to consider the possibility that this species, otherwise so unique in its habits of sexual reproduction, may never engage in cross-copulation. So far, only negative evidence can be brought to bear: we have never observed copulation, and we have established the fact that copulation between two individuals is not indispensable to vigorous sexual reproduction. Direct observations of copulation, or detailed breeding experiments involving genetically distinguishable individuals, are needed to settle this question.

SUMMARY

1. Experiments are described in which freshly-hatched juvenile specimens of *Curtisia foremanii* have been isolated in individual culture dishes and their ability to produce offspring in the absence of cross-copulation established. Some lines of these cultures, each worm of known pedigree from the P_1 generation, are now in the F_3 generation after approximately 9 months.

2. In the course of the experiments, indications have been noted that litter-mate worms may differ in the rate at which they attain maturity (criterion: deposition of first cocoon) and in their reproductive potential, and that strain differences in these qualities also exist in worms of diverse ancestry.

3. Although other species of fresh-water triclad Turbellaria have been reported as depositing only infertile cocoons in isolation, the overall percentage of infertility in Curtisia cocoons produced by isolated individuals is only 12%.

4. It has thus been demonstrated that cross-copulation is not essential to sexual reproduction in this species. Indeed, copulation of *Curtisia foremanii* has never been observed in this laboratory, and the possibility is suggested that it may never occur in this species.

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CALCIFICATION IN MOLLUSCS. III. INTAKE AND DEPOSITION OF Ca^{45} AND P^{32} IN RELATION TO SHELL FORMATION[1]

GERRIT BEVELANDER

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The problem of shell formation in molluscs has been the subject of numerous studies in the past and it is now generally conceded that there are two distinct phases which occur during the elaboration of the shell: (1) the formation of a protein membrane (periostracum); and (2) the mineralization of this membrane which results in the production of the calcified exoskeleton. It is generally agreed that the protein complex which becomes incorporated into the shell is elaborated by the distal portion of the mantle. Some of the details of this process are still somewhat obscure. Similarly, the factors involved in the deposition of calcium carbonate are also in several respects still subject to conjecture and speculation.

In a comprehensive review dealing with calcification in molluscs, Robertson (1941) has summarized a number of concepts which attempt to explain calcification in these organisms: Calcium carbonate is separated from the blood by certain cells at the mantle edge. The carbonate in a colloidal form is then liberated between the epithelium and the periostracum and subsequently undergoes crystalization in this matrix.

According to DeWaele (1930) the calcium carbonate of the shell is separated from the blood and from a so-called extra-palleal fluid, which, it is held, is a solution containing protein, carbon dioxide and calcium carbonate. By physico-chemical processes, the calcium carbonate forms as a result of the escape of carbon dioxide. Robertson further states that the greater part of the calcareous material of the shell in marine molluscs is absorbed directly from sea water as Ca and bicarbonate ions. This author's own experiments, however, fail to show a direct uptake of calcium ions from sea water in a number of gastropods and bivalves. It has further been suggested by Baldwin (1935) and by Freeman and Wilbur (1948) that metabolic carbon dioxide may be the primary source of the carbonate radical.

In regard to the source of calcium which is utilized in the formation of the shell, Orton (1925) observed that shells continue to grow in the English oyster in the absence of food. Similar observations were recorded for the American oyster by Galtsoff (1934) who states in this connection that the amount of calcium utilized is many times greater than could be stored in tissues. Fox and Coe (1943) in their studies on Mytilus also deduce from their observations that the amount of calcium obtained from food alone does not account for the amount deposited in the shell. It has also been shown (Bevelander and Benzer, 1948) that when the calcium

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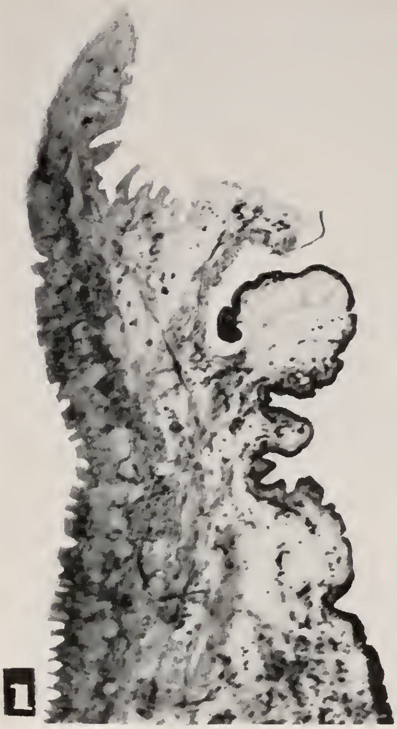
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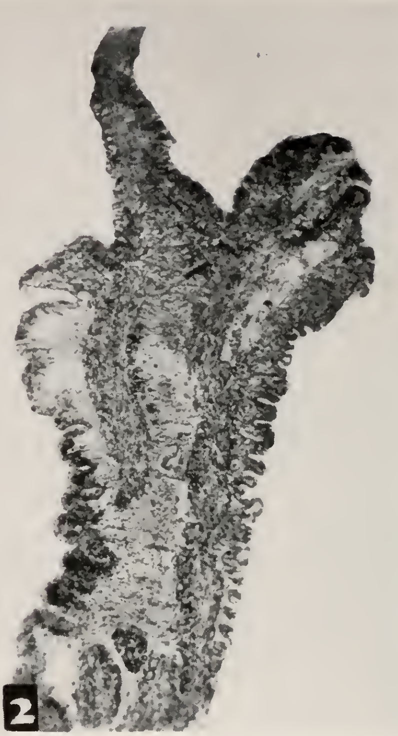
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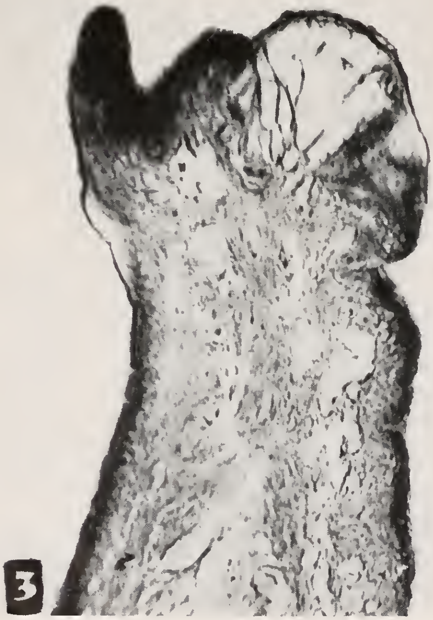
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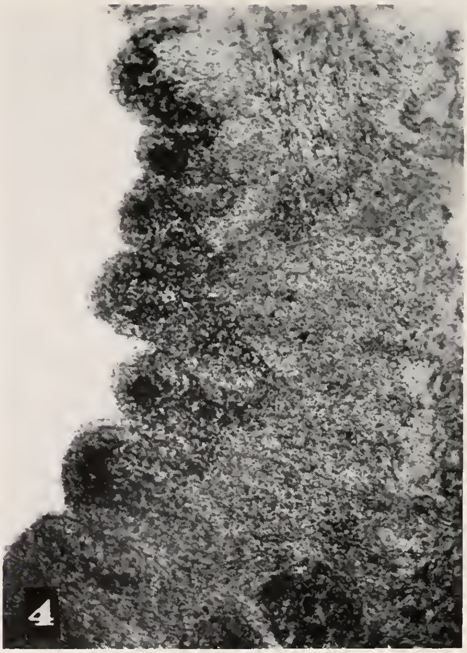
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content of sea water is reduced, a corresponding reduction in the calcium of the growing shell results.

From the foregoing it is clear that a number of factors involved in the production of shells in molluscs are subject to controversy. Further, many of the ideas proposed to account for calcification are based upon indirect evidence.

This report will deal primarily with the source, transport and deposition of Ca^{45} and P^{32} in the mollusc with special reference to the relation these minerals bear to the formation of the shell.

Materials and Methods

Several species of marine and fresh water molluscs were utilized in this investigation. For study of localization of radioactive salts in the mantle, the specimens were placed in a large aquarium to which tracer amounts of the appropriate isotope were added. The water was constantly agitated and the specimens were allowed to remain in water for periods ranging from two to twenty-four hours.

After the specimens were removed from the aquaria, the mantle was removed and processed for sectioning. The tissues were subsequently mounted on photographic film which was exposed for various times and then developed.

Pieces of shell which were used in making radio-autographs were obtained from specimens of Pinna which had been induced to regenerate shell in sea water to which Ca^{45} or P^{32} had been added. The pieces were removed from the experimental animals and after numerous washings the shell fragments were thoroughly dried and placed on photographic film. Following exposure, the films were developed as previously described.

Other procedures followed in connection with the preparation of sections for histological and histochemical studies have already been described (Bevelander and Benzer, 1948) and need not be detailed here.

Experimental

Mantle:

The localization of Ca^{45} in the mantle of both fresh water and marine molluscs was found to be essentially similar. In the radio-autograph of the mantle of *Anodonta grandis* shown in Figure 3, one can observe that calcium is localized on the entire periphery of the mantle. The calcium appears to be confined to a region slightly below the surface epithelium and also in the region of the epithelium itself. Further, the tentacles appear to have a more extensive zone of calcium storage than the more proximally located parts.

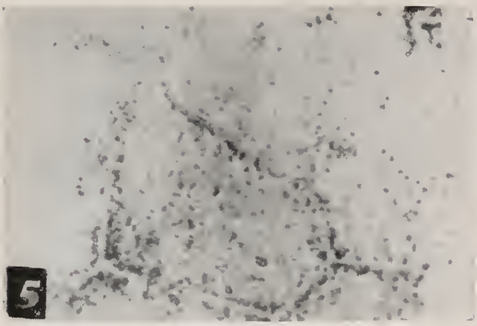
The localization of P^{32} in the mantle is illustrated in Figures 2 and 4. Figure 2, a low power photograph of the mantle of Venus, shows that P^{32} is confined pri-

FIGURE 1. Longitudinal section of mantle of *Anodonta grandis.* Black-staining margin on right side of mantle indicates localization of alkaline phosphatase.

FIGURE 2. Radio-autograph of longitudinal section of mantle of Venus. Localization of P^{32} is shown by blackened sub-epithelial areas on left side of mantle.

FIGURE 3. Radio-autograph of longitudinal section of the mantle of Anodonta. Note blackened areas on the periphery of the mantle which indicate the localization of Ca^{45}.

FIGURE 4. High power area of mantle illustrated in Figure 2, to show more clearly the localization of P^{32} in the sub-epithelial zone.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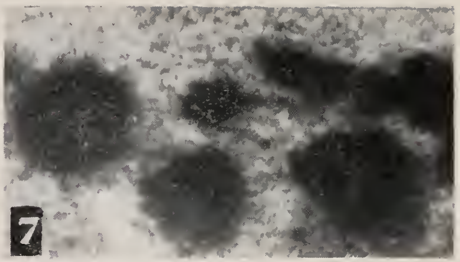
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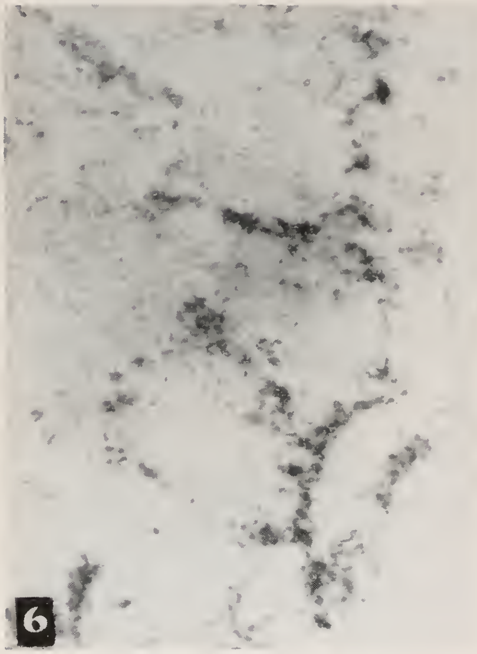
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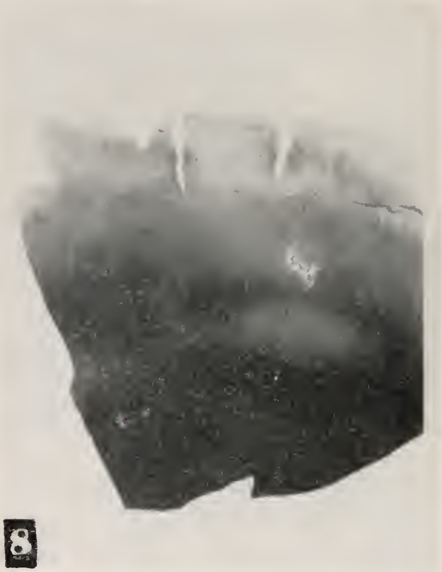
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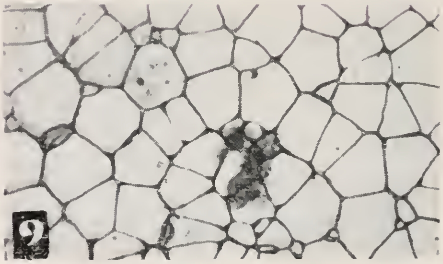
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FIGURES 5 AND 6. Radio-autographs of crystals of the shell of Pinna to show localization of P^{32} in the periostracum.

FIGURE 7. Radio-autograph of crystals of Pinna showing localization of Ca^{45} within the crystals. Extraneous blackening of photo due to over-exposure.

FIGURE 8. Large fragment of shell of Pinna showing differential uptake of Ca^{45} in regenerating shell.

FIGURE 9. Surface view of shell of Pinna to show relation of crystals (light areas) to the surrounding periostracum shown as dark lines surrounding each crystal.

marily to the inner surface of the mantle. Examination of Figure 4, which is a photograph of this same area taken at higher magnification, shows that P^{32} is localized in regions of the mantle below the surface epithelium. The P^{32} observed in the mantle occupies a position in this organ which corresponds to the location of the mucus glands. Inasmuch as the method of processing the tissues involved several exposures to aqueous solutions, a procedure which tends to eliminate water-soluble phosphate from the tissues, it appears that the phosphate which remained in the mantle which was subsequently localized in the radio-autographs is an organic phosphate complex.

The sites in which alkaline phosphatase occurs in mantle tissues of molluscs were also ascertained in a variety of species. The localization of this enzyme in the mantle is fairly well exemplified in the fresh water specimen Anodonta which we have chosen to illustrate. Reference to Figure 1 shows that phosphatase is present on the entire inner surface of the mantle. The enzyme is further confined for the most part to the epithelial cells which form the surface of the mantle. The numerous amoebocytes found in this structure also reveal the presence of phosphatase.

Shell:

The localization of Ca^{45} in the shell of Pinna which was laid down under the experimental conditions already described, is shown in Figures 7 and 8. Figure 8 is a reproduction of a radio-autograph of a fairly large piece of regenerated shell which shows an overall black appearance which is lighter in the thinner and more recently formed margin. When selected regions of this shell are examined microscopically, one observes that the Ca^{45} is confined to the areas occupied by the crystals (Fig. 7), while the adjacent peripheral area occupied by the periostracum (see Fig. 9) is relatively devoid of Ca^{45}.

The identification of P^{32} in the regenerating shell of Pinna was ascertained in a manner similar to that which was utilized in connection with Ca^{45}. Reference to Figures 5 and 6 showing several crystals of the shell of Pinna grown in the presence of P^{32} indicates the precise localization and the details of structural variation of the crystal. Insofar as one can evaluate by the method utilized, P^{32} comes to be localized in the protein membrane (periostracum) which encloses the crystals. The effect thus produced by the introduction of P^{32} in the growing shell examined by radio-autographic methods is virtually a negative image of the picture produced by the addition of Ca^{45}.

DISCUSSION

It was pointed out in our introductory remarks that the elaboration of shells in the mollusc consists in the formation of a protein membrane, the periostracum, and a concomitant or subsequent mineralization of this membrane. From observations based upon several studies made on the organic component of the shell, it appears highly probable that this protein complex may be actively involved in the formation or growth of the mineral crystals which come to be incorporated in the shell. Since our observations on this latter topic are still incomplete we have for the present confined our remarks to some aspects of shell formation which are concerned more intimately with the role of some inorganic constituents of the shell.

Although several studies suggest that the calcium utilized in the formation of the shells of marine molluscs is derived in part from the sea water in which the animals live (Orton, 1925; Galtsoff, 1934; Fox and Coe, 1943), a number of factors in regard to the source, transport and utilization of calcium still remain obscure.

By using labeled calcium we have shown that calcium ions present in the water are ingested by the organism and are localized in several organs. Of particular interest is the observation that relatively large amounts of labeled calcium are concentrated on the periphery of the mantle. A somewhat similar observation has been recorded by Haysi (1938) and also by Trueman (1942). The method of converting the calcium to calcium oxalate as used by these authors, however, is capricious and subject to error in localizing the calcium crystals.

It was also shown that the labeled calcium which was placed in the water (as calcium chloride) was rapidly incorporated into the mineral component of the shell. Control experiments, by means of which detached pieces of shell were placed in the Ca^{45} solutions, did not reveal the localization of Ca^{45} in the crystals. It is apparent, then, that the mollusc can and does utilize calcium in an ionic form in the production of the mineral component of the shell.

The localization of phosphate and phosphatase in the mantle of the mollusc is not only intriguing but is also a tempting subject upon which to speculate. Manigault (1939) has stated that a direct correlation exists between the mantle phosphatase activity and calcium precipitation in the shell, and further, that phosphatase serves as a transfer agent in the mobilization of calcium. The validity of this concept still awaits confirmation. It has been shown that phosphorylation does occur as a result of the action of mantle phosphatase upon mantle mucus (Bevelander and Krimsky, 1949). The significance of this reaction in relation to shell formation, however, is at present unknown.

Trace amounts of phosphorus have been recorded for mollusc shells (Turek, 1933). It seems fairly evident from our observations that the phosphorus in the shell is confined to the organic constituent of the exoskeletal complex.

SUMMARY

1. Both fresh water and marine molluscs take up labeled calcium and phosphorus from the water.

2. Labeled calcium is concentrated on the periphery of the mantle and also is incorporated into the crystals of calcium carbonate in the newly formed shell.

3. Labeled phosphate was localized on the inner margin of the mantle in the region of the mucus glands. It was also incorporated in the periostracum surrounding the crystals.

4. The phosphatase and phosphate present in the mantle are concerned with a phosphorylating process; the significance of this phenomenon in relation to mineralization is not clear.

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REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE BY MITOTICALLY ACTIVE AND BLOCKED EMBRYONIC CELLS[1]

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It has been reported by many workers that the reduction of water-soluble colorless triphenyltetrazolium chloride (T.T.C.) to its insoluble red formazan is indicative of physiologically active reductases and thus can give some idea of the metabolic activity of a living tissue or homogenate (Black and Kleiner, 1949). The apparent redox potential of the dye in an aqueous solution at pH 7 was reported by Jerchel and Möhle (1944) to be − 0.08V. Mattson, Jensen and Dutcher (1947) reported that glucose dehydrogenase reduced this compound and suggested that the reduction involves dehydrogenase systems requiring Co I and Co II. They suggest further that the compound may act as an electron acceptor for many pyridine nucleotides, for the redox potentials of these compounds fall below − 0.08V. Kun and Abood (1949) reported that T.T.C. could be used as an indicator of succinic dehydrogenase in animal tissue homogenates. Brodie and Gots (1951) showed the reduction of T.T.C. by isolated yeast diaphorase systems. Fred and Knight (1949) found a slowing of the reduction by metabolic depressants, sodium azide, sodium malonate, sodium fluoride, 2,4-dinitrophenol and iodoacetate in *Penicillium chrysogenum*. They also reported that aeration by shaking retarded reduction and suggested that oxygen possibly raised the redox potential above − 0.08V or that oxygen competed with the indicator. Roberts (1951) suggests the involvement of sulfhydryl-containing enzymes in the reduction of T.T.C. in plant meristems.

In view of the growing evidence that the reduction of T.T.C. seems indicative of an over-all metabolic rate, the factors responsible for its reduction in the mitotically active and blocked grasshopper embryos were investigated and data obtained are embodied in this report.

Material and Methods

Grasshopper (*Melanoplus differentialis*) embryos of known developmental and temperature histories were dissected from eggs in Ringer solution buffered with 0.66 *M* phosphate to a pH of 6.8. Mitotically active embryos (pre- and post-diapause) as well as those blocked (diapause) were employed. One hundred embryos per ml. of suspension media were used in all experiments. Homogenates from intact embryos were prepared both in Ringer and 0.25 *M* sucrose as previously indicated (Bodine and Lu, 1950). All experiments with the dye were conducted at 42° C. unless otherwise specified. This temperature was found most adequate for satisfactory reduction of the dye within a one-hour period. Both

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[1] Aided by a grant from the National Institutes of Health. Grateful acknowledgment is made to Etta Andrews for technical assistance.

embryos and dye were allowed to reach this temperature before mixing. During exposure to the dye the tubes containing the experimental materials were shaken at a slow constant rate to ensure thorough mixing of dye with embryos. At the end of a one-hour period the aqueous solution was decanted, the embryos washed and then 1 cc. of acetone added. The red formazan was further extracted by repeated washing with iso-amyl alcohol to a final volume of 5.5 cc. The amounts of formazan recovered were then checked with standards previously prepared. Determinations were carried out with the Coleman Model D spectrophotometer at 575 mμ, and pc-4 filter. With homogenates it was necessary to centrifuge tubes in order to throw down interfering particles before making readings.

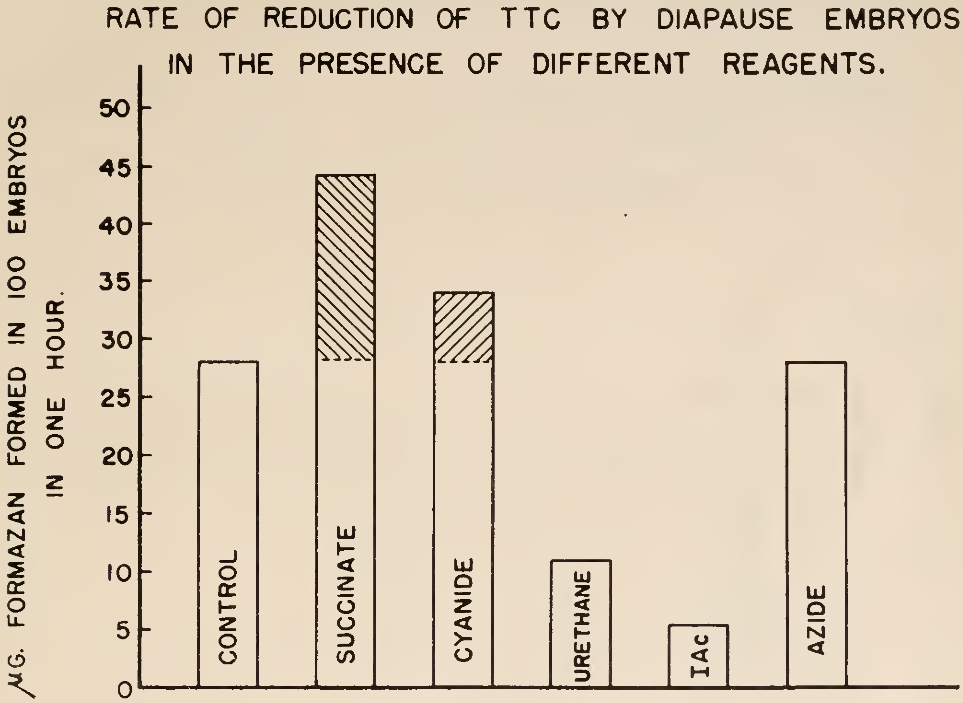


FIGURE 1. Shows average amounts of formazan formed in 1 hour at 42° C. by 100 diapause embryos. Control in Ringer. Succinate, — 0.06 *M*; cyanide, 1×10^{-4} *M*; iodoacetate, 0.01 *M*; sodium azide, 1.6×10^{-3} *M*.

Experiments were also carried out using Thunberg tubes in order to check qualitatively results obtained by the former method. In all experiments 0.1 per cent solution of T.T.C. was employed.

RESULTS

Incubation of the embryos for one hour at 42° C. in 0.1 per cent T.T.C. stains them an intense pink readily discernible with the naked eye. When examined cytologically the red formazan is found to be concentrated in both the inter- and intracellular lipids suggesting a differential lipid solubility of the dye. The intensity of the red color appears to vary directly with the length of exposure to the dye and the temperature of the medium. At 42° C., a one-hour exposure seems to produce the maximum color. Lower temperature, 25° C., takes longer periods of time for maximum color production. If intact embryos or homogenates are heated at 60° C. for periods of 20 minutes, no reduction of the dye is observed, suggesting the destruction of the inherent reducing enzymes at this temperature. When homogenates are made from intact embryos and dye added, the production of the red formazan at room temperature is much retarded. Apparently structure or intactness of the embryo controls to some extent the degree to which reduction of the dye can take place. Homogenates made in 0.25 *M* sucrose appear to give quicker reduction of T.T.C. than when prepared in Ringer. Results for intact embryos and the amount of formazan produced are graphically summarized in Figure 1. Normal embryos (mitotically active) produce approximately 28 μg. of formazan in one hour at 42° C. from a 0.1 per cent solution of the dye. During diapause or in mitotically blocked embryos the amounts of formazan produced tend to be lower than those found for pre- or post-diapause (mitotically active) stages and would seem to indicate a more active reducing capacity on the part of the cells when mitotically active (Fig. 2). This point, however, needs further careful study before definite conclusions are possible.

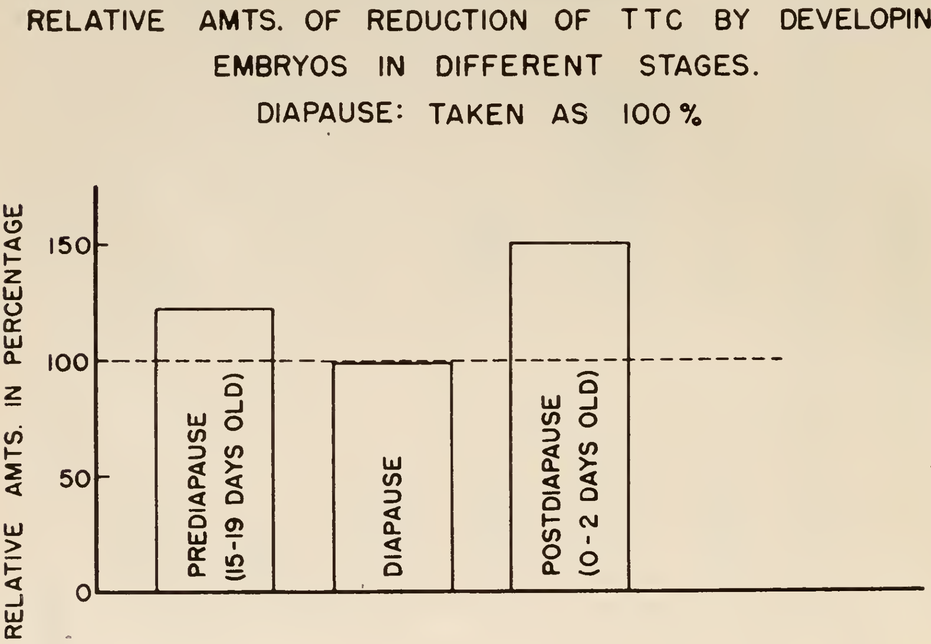


FIGURE 2. Shows relative amounts of reduction of T.T.C. by embryos in different stages. Diapause taken as 100 per cent.

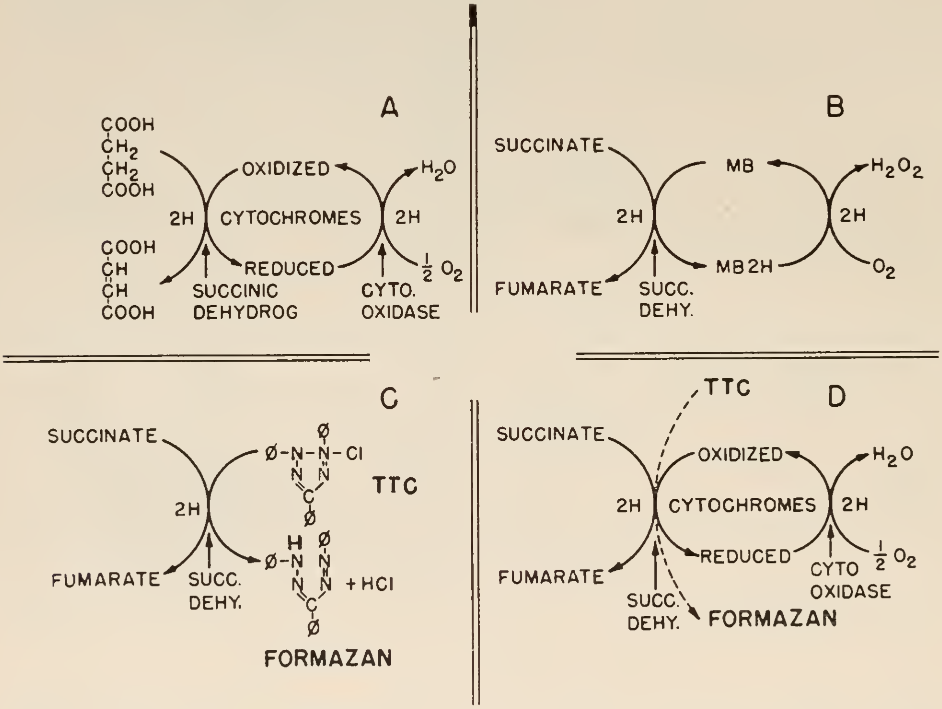
Our data seem to indicate that the reduction of T.T.C. by grasshopper embryos is quite non-specific as far as definite reducing systems are concerned. Certain metabolic inhibitors known to penetrate the intact embryos were added and their effects upon the formazan followed. Iodo-acetate (0.01 *M*), an inhibitor of sulfhydryl-containing enzymes, completely depresses the reduction of the dye (Fig. 1). Roberts (1951) and Fred and Knight (1949) report a slowing of T.T.C. reduction by this reagent in physiologically active systems. Urethane (0.3 *M*), a general dehydrogenase depressant, depresses T.T.C. reduction in the embryo (Fig. 1). This is contrary to results reported by Roberts (1951). Potassium cyanide (1×10^{-4} *M*) and sodium azide (1.6×10^{-3} *M*) apparently do not have any marked effect on T.T.C. reduction at these concentrations. As a matter of fact a slight stimulation is found for cyanide (Fig. 1).

SYSTEM	TEMP.	COLOR INTENSITY AFTER 90 MINS.
TTC + Homogenate	25°C	±
" "	42°C	±
" " + Succ.	42°C	+
THUNBURG METHOD		
TTC + Homogenate	25°C	+
" " + Succ.	25°C	+ + +
" + Supt. Fraction	25°C	+
" " + Succ.	25°C	+ + +
M.B "	25°C	(Colorless After 3.5 Hours)
" " + Succ.	25°C	(Colorless After 90 Min.)

TABLE I. Shows comparative results obtained by aerobic and anaerobic methods. ± little if any color; + faint pink; + + + intense pink.

Dehydrogenase activity of intact embryos is reduced to a greater extent by homogenization than the endogenous oxygen uptake (Bodine, Lu and West, 1951). The homogenate T.T.C. system did not show a positive response until approximately 90 minutes (very slightly pink) (Table I) and reduction in this system has been studied only qualitatively.

Thunberg showed that the dehydrogenase activity of physiologically active systems was responsible for the reduction of redox dyes. In a comparison of the methylene blue-supernatant fraction (cytoplasm) with that of T.T.C.-supernatant fraction by the Thunberg method, reduction was observed in both instances (Table I). The speed of reduction was stimulated by the addition of sodium succinate (0.06 *M*). Methylene blue has been shown to have no effect on the endogenous oxygen uptake of the supernatant fraction (Bodine and Lu, 1950). Potter (1951) suggested that the electron turnover rate is faster for cytochrome than methylene blue. Thus, in a cytochrome-rich system (homogenate) the characteristic stimulatory effects may not be seen manometrically (O_2 uptake) (see chart). Bodine *et al.* (1951) found a lowering of both endogenous and succinate-stimulated respiration (O_2 uptake) by the addition of T.T.C. Malonate studies now in progress reveal little or no succinic dehydrogenase involved in the endogenous respiration of homogenates. Thus it appears that T.T.C. reduction in the grasshopper embryo probably involves the sum of several physiologically active reductases.


A
COOH
CH2
CH2
COOH
OXIDIZED
H2O
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CYTOCHROMES
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SUCCINIC
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FUMARATE
OXIDASE
SUCC.
DEHY.
FORMAZAN

FIGURE 3. Shows schematically probable method of working of T.T.C. in cytochrome system.

SUMMARY

1. A study has been made of the reduction of triphenyltetrazolium chloride by the mitotically active and blocked embryo of the grasshopper, *Melanoplus differentialis*.

2. Reduction by mitotically active embryos seems more active than by blocked ones.

3. Present data seem to indicate non-specific reducing systems involved in the reduction of the dye.

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ANAEROBIC AMMONIA PRODUCTION BY AMPHIBIAN GASTRULAE EXPLANTS

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Boell, Needham and Rogers reported (1939) that dorsal lip explants of *Rana temporaria* gastrulae, under anaerobiosis, produce some three times as much ammonia as ventral explants under similar conditions. Their finding is of considerable interest, for it is just this sort of metabolic correlate to gross morphology that chemical embryologists hope to discover. But attempts to confirm their results, together with a close examination of their experimental procedures, have convinced us that there is little basis for their claim.

NOTES ON METHODS

Explants: Dissections were made with glass needles in the usual manner. Explanted pieces were allowed to heal well in 100% Holtfreter's before beginning the experiment. Great care was taken to prevent contact of any explant with air-water interfaces—hence in no experiment was there any sign of cytolysis.

Weighing: Explants or brei (25–90 μg. dry weight) were pipetted on to small bits of cigaret paper, dried for one-half hour at 100° C., and weighed immediately on a quartz helix balance to ± 1 μg.

Anaerobiosis: Explants or brei (ca. 1 mg. dry weight) were placed in 24–25 μl. of 100% Holtfreter's solution (with or without bicarbonate) in small aluminum-foil dishes. The dishes (blanks and experimentals) were set in a closed horizontal glass cylinder with a stopcock at each end. The gas mixture (95% N_2-5% CO_2) was run through the cylinder for a half hour after first being passed over a hot copper screen and through a water wash. (In two cases, hydrogen gas was used, de-oxygenated with hot platinized asbestos.) The stopcocks were then closed for the duration of the experiment (usually 3–4 hours). Under these conditions, methylene blue barely reduced with hydrosulfite did not change color for at least 18 hours; hence, anaerobiosis was at least as complete as in the experiments of Boell *et al.*, who used a similar criterion. Aerobic controls were kept in covered dishes in air.

Temperature: All experiments were conducted at room temperature (20–25° C.).

RESULTS

When whole gastrulae (*Rana pipiens,* stage 10, each weighing dry about 1 mg.) are cut into about 20 explants and placed at room temperature in Holtfreter's solution, they excrete traces of ammonia (all experiments were controlled with Holtfreter's blanks) into the surrounding medium:

(1) 0.45 ± 0.21 μg. NH_3/gastrula/5 hours (4 expts., aerobic).

Similarly treated gastrulae under anaerobiosis (*cf.* Notes on Methods) gave similar results:

(2) 0.20± 0.17 μg. NH_3/gastrula/5 hours (7 expts., anaerobic).

We do not attach any significance to these values other than that they indicate the excretion of small traces of ammonia. Our ammonia method (Brüel *et al.*, 1946) had an upper range of about 7 μg. NH_3. Under our routine conditions, standard NH_3 samples are recovered with a reproducibility of about ± 2% of the total range, *i.e.*, ± 0.14 μg. NH_3. With this method, the ammonia present in the aliquots available for analysis—up to 0.13 μg., corresponding to that produced during the experimental period by about 300 μg. tissue—was barely detectable.

The results of Boell, Needham and Rogers are not much better off in this respect. From their account, it is easy to calculate that their ammonia method (similar to ours in all essential respects) had an upper range of about 3 μg. NH_3. We shall make the fair assumption that the reproducibility of their determinations was ± 2% of this range, *i.e.*, ± 0.06 μg. NH_3. A few simple calculations from their data and the description of their experimental procedure show that in the case of the dorsal lip explants (their Table IV) the average aliquot actually taken for ammonia determination contained only about 0.18 μg. NH_3, and that in the case of the ventral explants (their Table V) the average aliquot contained only about 0.075 μg. NH_3. Since they must have been working almost at the limit of reproducibility of their method, it is clear that, like ours, their results:

(3) Dorsal lip: 1.75 μg. NH_3/mg. dry wt./5 hours

(4) Ventral: 0.61 μg. NH_3/mg. dry wt./5 hours

have little quantitative significance. ((3) and (4) have been recalculated from their tables so as to be comparable to our results (1) and (2).)

But to assure ourselves that we were not failing to confirm their results simply because our ammonia method was too insensitive, we scaled it down so that the upper range was approximately 1 μg. NH_3. With this method, the anaerobic NH_3 production by pooled dorsal halves of 3 gastrulae was:

(5) 0.073 μg. NH_3/mg. dry wt./5 hours;

for the corresponding ventral halves, the production was:

(6) 0.22 μg. NH_3/mg. dry wt./5 hours.

Even with the scaled-down method the ammonia in the aliquots (corresponding to that produced by about 300 μg. dry wt. tissue—many times more tissue than employed by Boell *et al.*) was too low (0.017–0.068 μg. NH_3) for accurate measurement; therefore neither the relative nor the absolute magnitudes of the values in (5) and (6) are to be taken seriously.

Nor does the difference between our claim and that of Boell *et al.* appear to be the outcome of studying gastrulae of different species: a dissected *Rana temporaria* gastrula put through our standard procedure failed to produce any ammonia anaerobically, and another failed to excrete any ammonia aerobically in four hours (in both cases, actually, the blank controls were slightly higher than the experimentals). (These gastrulae were kindly furnished us by Professor John A. Moore.)

Not only do gastrulae explants fail to excrete much ammonia into the circumambient medium, but they appear not to produce it *at all* in any significant amounts; for when explants (dissected whole gastrulae) at the end of the experimental period, whether aerobic or anaerobic, are ground up and precipitated with tungstate, the supernatant still contains only traces of ammonia:

(7) 0.50 ± 0.11 μg. NH_3/gastrula/5 hours (aerobic, 3 expts.);

(8) 0.30 ± 0.11 μg. NH_3/gastrula/5 hours (anaerobic, 3 expts.),

indicating that we do not fail to find ammonia in the medium around explants simply because the tissues bind it in some way as fast as it is produced.

But when *breis* are taken for ammonia analysis, large amounts of ammonia are obtained (presumably the strong alkali used in the method hydrolyzes ammonia-containing compounds in the tissues):

(9) 13.2 ± 0.25 μg. NH_3/gastrula (3 expts.).

Now, (9) provides us with further grounds than those already advanced for casting grave doubt upon the significance of the claims of Boell *et al.* For it is quite likely that their explants underwent considerable cytolysis during their experiments (*cf.* Brachet's discussion of this point, 1950, pp. 375–376); and this means that in their analytical samples there must have been considerable ammonia-liberating cytolysate. We can understand, then, (a) why they seemingly found more ammonia to be excreted than they might have expected from their results on intact gastrulae (*cf.* their remarks: Boell *et al.*, 1939, p. 352); (b) why the dorsal explants seemed to produce more ammonia than the ventral explants, for it is well known that dorsal explants are more sensitive than ventral explants to all kinds of cytolyzing influences (we have calculated on the basis of (9) that about an 8% cytolysis of their dorsal explants would have accounted for the reported difference from the ventral explants in NH_3-production); and (c) why they found that NH_3-production of dorsal tissue seemed to decrease with increasing age of their embryos (*cf.* their chart; Boell *et al.*, 1939, p. 348), for it is common knowledge that as embryos develop, their resistance to cytolysis in air-water interfaces increases tremendously.

Taken all together, the evidence almost forces us to conclude that although gastrulae explants excrete traces of ammonia both aerobically and anaerobically, these are so small as to be beyond the effective range of available ammonia methods; and that to date no quantitative statement about relative rates of ammonia excretion by different gastrula-parts has been experimentally justified. But if this is the case then the anaerobic glycolysis values of the Cambridge embryologists (Boell *et al.*) need re-evaluation also; for they were corrected for lactate allegedly bound by ammonia differentially excreted by dorsal and ventral explants.

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THE COMPARATIVE EFFECTS OF FISH AND BEEF PITUITARY ON RETENTION OF WATER BY FROGS

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Amphibians treated with the posterior lobe of mammalian pituitary greatly increase in weight due to the imbibition and retention of water. Two fractions from the lobe, pitocin and pitressin, will induce the effect (Bugbee and Kamm, 1928). The results are caused by a change in the permeability of the skin, allowing the intake of more than the usual amount of water, and by the storage of the excess water in muscles, liver, and subcutaneous spaces (Adolph, 1925; Heller, 1930a). Apparently there is also an associated anti-diuretic action (Heller, 1950). Most studies relating to this principle of the pituitary have been made with bovine glands or their extracts, but it has also been shown to be present in the pituitaries of such teleosts as shiners, catfish, perch, sunfish, rockbass, and cod (Boyd and Dingwall, 1939; Heller, 1941). In this investigation, experiments were undertaken to see whether or not the glands of the sockeye salmon and the Pacific barracuda induce a similar effect, and, if so, to compare statistically their activity with beef pituitary.

MATERIALS AND METHODS

Inasmuch as the lobes of the teleost pituitary are small and united, all tests and comparisons were made with whole glands. It is believed that the outcome was practically the same as though posterior lobe alone were used. Heller (1930b) stated that anterior lobe preparations do not cause any weight changes and that the action of the posterior lobe hormones is not affected by their presence. As for the intermediate lobe, Oldham (1936) reported that the chromatophorotropic hormone does not enter in the reaction.

Portions of brain from fish and cattle were used as control substances. Fresh fish pituitary and brain were taken from sockeye salmon (*Oncorhynchus nerka*) during their spawning run at Flathead Lake, Montana. These were kept frozen until time of use. Acetone-extracted fish pituitary and brain, which were ground to a fine powder, desiccated, and sealed under reduced pressure in glass ampoules, came from barracuda (*Sphyraena argentia*). Brain and whole pituitaries of beef were extracted in the same way.

Medium-sized *Rana pipiens* served as test animals. The general procedures followed in treating them were similar to those of Steggerda (1937) and Boyd and Brown (1938). The frogs were placed individually in gallon jars with enough tap water to cover all the body surface but the eyes and nostrils. Porous cardboard covers excluded air drafts, which according to Boyd and Mack (1940) is a factor to be considered. The illumination was constant artificial light. Water temperatures varied from 18–25° C., but there was no greater change than 1° from the

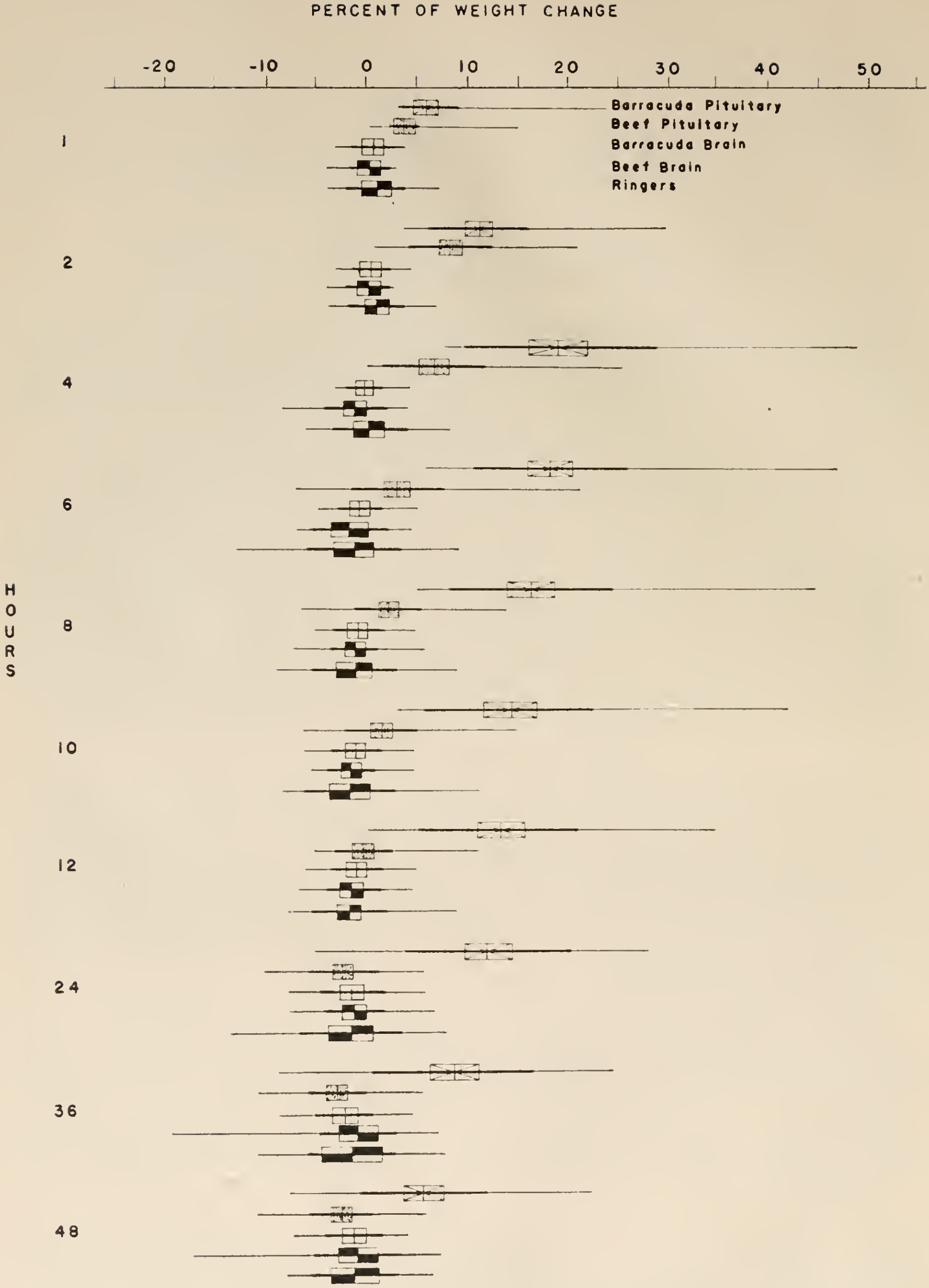

FIGURE 1. Graph of weight changes of frogs injected with barracuda and beef pituitary extracts as compared with those injected with an equivalent amount of barracuda brain extract, beef brain extract, and pure Ringer's solution, drawn according to the method of Hubbs and Perlmutter (1942). The range for each group is indicated by the length of the light line, the position of the mean is shown by a crossbar, the open rectangle marks off two times the standard error on each side of the mean, and the heavy line extends for one standard deviation on each side of the mean.

start to the finish of an experiment. The frogs were acclimated to this environment for 24 hours before they were tested.

The extracted and fresh materials, suspended in 0.5 cc. Ringer's solution, were injected into the dorsal lymph sac. Frogs tested with fresh salmon pituitary received 2 whole glands, and their controls a comparable amount of fresh salmon brain. Desiccated extracts were all given in doses of 4 mg. Immediately after injection, each frog was dried with paper toweling, had its urine expelled as much as possible by pressure on the abdomen, and was then weighed. The weighing was repeated at regular intervals until the frog had regained its normal weight. Most of the test animals were utilized twice. Those which had been treated with pituitary were re-injected a week later with one of the control solutions, and those which had been control animals received one of the pituitary preparations. It was believed that by doing so, more accurate evidence would be obtained, as the variability in individual reaction would be included in both experimental and control groups.

The ponderal changes were figured on a percentage basis and analyzed statistically. The statistical comparisons between means followed the method of Dice and Leraas (1936), in which any difference between two means which is greater than two times the sum of the standard errors of the two respective means is considered to be significant.

RESULTS

All frogs injected with any of the pituitary preparations markedly increased in weight. Fifty specimens which received the fresh salmon gland had an average increase of 16.0 per cent 4 hours after treatment. This rapid increase was followed by a gradual decrease to 2.8 per cent 32 hours later. The arrival of the mean weight to within 2.8 per cent of their original weight was considered a return to normal, as the weight variation for a group of untreated frogs had been previously determined to be plus or minus 4.2 per cent. Forty-four frogs injected with 4 mg. desiccated barracuda pituitary showed an increase to 18.9 per cent in 4 hours, followed by a decline to 5.5 per cent 48 hours after treatment. The mean weight change of 44 frogs that received 4 mg. desiccated beef pituitary reached a maximum of 8.1 per cent 2 hours after injection. They returned to their original weight range within 10 hours.

The controls (19 injected with fresh salmon brain, 20 with desiccated barracuda brain, 20 with desiccated beef brain, and 21 with straight Ringer's solution) did not show any weight fluctuations outside the normal limits.

A statistical study of the changes induced by beef and fish gland extracts shows that the rise in weight was significantly different from those of the control groups, beginning 1 hour after treatment (Fig. 1). The effects of the two pituitary extracts were not significantly different from each other at this time. After the first hour, however, increases in weight caused by barracuda pituitary were greater than those caused by beef pituitary, and the effectiveness was longer lasting. The frogs treated with beef pituitary extract remained significantly heavier than controls for 10 hours, whereas those which received barracuda pituitary extract retained a significantly greater weight than the controls for over 48 hours.

Discussion

It is probable that the increases and decreases in weight shown by the control groups represent a normal, random fluctuation. These changes may be partly attributed to retention or loss of urine. The fact that their average weight was less towards the end of an experimental period as compared with the first part may be explained by the fact that their first weights were determined immediately after injection of material suspended in 0.5 cc. of saline solution. The complete expulsion of this liquid from the body could account for a decrease in weight of from 2.0 to 5.0 per cent, depending on the size of the animal.

On the other hand, the series injected with pituitary exhibited a fairly definite pattern, namely, a rapid increase in weight in 2–4 hours, followed by a gradual decrease to normal.

The similarity in potency of fresh fish pituitary and desiccated fish pituitary indicates that the process of extraction did not materially change the effect of the hormone. Inasmuch as the amount of fresh gland only roughly approximated the 4 mg. of desiccated materials used, and as the rate of absorption must have been different, it is not possible to collate its activity.

There are three marked differences between frogs treated with desiccated beef pituitary and those treated with desiccated fish pituitary. (1) The beef extract induced its peak effect earlier than the fish extract. (2)The fish extract caused a greater maximum weight gain. (3) The fish extract had a more prolonged effect.

The first difference in particular may be explained partly by the dissimilarity in the size of the particles of the injected powders. Since the fish material was the coarser of the two, it may have been absorbed more slowly. This would also help to account for the third difference.

The second and third divergences strongly suggest that teleost pituitary is more potent in its effect to cause water absorption and retention in amphibians than is bovine pituitary. This is in agreement with Heller's (1941) findings that the pituitaries of mammals contain large amounts of an anti-diuretic principle but relatively little of the amphibian water retention factor, whereas amphibian and fish glands contain high concentrations of the water retention principle but have little anti-diuretic activity. In an equal amount of extract, there must be either a greater concentration of the principles (principle?) in the fish gland, or the factors concerned in the pituitaries from these two widely separated vertebrates may differ basically. Also, a closer phylogenetic relation of teleosts to amphibians than of mammals to amphibians is indicated.

Although Boyd and Dingwall (1939) reported that injected pituitrin did not change the weight of the five species of fish they tested, there is still the interesting possibility that marine teleosts, which live in a hypertonic medium, may possess a hormonal mechanism in the pituitary that can greatly inhibit water loss. In this respect, the glands of marine and fresh-water species should be compared.

Summary

1. Frogs injected with fresh salmon pituitary, whole beef pituitary extract, or whole barracuda pituitary extract undergo a rapid increase in weight followed by a more prolonged phase of weight decrease to normal. Controls treated with fresh

salmon brain, bovine or barracuda brain extracts, or with Ringer's solution alone do not exhibit weight changes outside the normal fluctuations.

2. A statistical analysis of weight changes induced by the teleost and beef pituitary extracts shows a highly significant difference between the two; the fish material causes a greater and more prolonged weight increase.

3. The greater potency of the fish material may be due to a difference in the concentration of the principles involved, or the principles may differ basically.

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OBSERVATIONS ON REPRODUCTION OF THE POECILIID LEBISTES RETICULATUS (PETERS)

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Reproduction among members of the viviparous cyprinodont fishes has stimulated the interest of investigators for many years. Various members of the family have been reared in home aquaria and, for the most part, general facts concerning the size of broods, interval between broods, cyclical nature of brood production, rate of growth of the embryo and the time of fertilization of ova in relation to the birth of a previous brood, are well known (Hopper, 1943; Scrimshaw, 1944, 1945; Tavolga and Rugh, 1947; Turner, 1937, 1940).

Knowledge concerning the reproductive cycles of these fishes has been obtained primarily by following the brood production of individual females, removal of the ovaries at intervals after the birth of a previous brood, and examination of the ovaries for the developmental stages of ovocytes and embryos. These studies have shown the brood interval to be approximately 30 days for *Xiphophorus, Platypoecilus* and *Lebistes* when maintained in aquaria under controlled conditions of temperature and light. Under natural conditions, brood production in these genera reaches a maximum during the summer months when the number of young per brood increases and the brood interval may be as short as 22 days. During the winter months the brood interval may lengthen to more than 30 days or brood production may cease entirely.

When these fishes are maintained in aquaria with controlled temperature and light, the gestation period in *Platypoecilus* is approximately 21–23 days, maturation of the ova occurring during the first 7 days after the birth of a brood (Hopper, 1943; Tavolga and Rugh, 1947). In general, *Xiphophorus* and *Lebistes* show a similar cycle as do the majority of viviparous cyprinodonts (Turner, 1937). The recent publication of Clark and Aronson (1951) includes a thorough discussion of the mating behavior of *Lebistes* and other members of the family.

As part of a study concerning the genetic mechanisms of the lordotic mutation (Rosenthal and Rosenthal, 1950; Rosenthal, 1951a), information was obtained on the reproductive behavior of male and female *Lebistes*. It is hoped that the results of this work will be helpful for a more complete understanding of reproductive physiology in these interesting fishes.

MATERIALS AND METHODS

The majority of fishes used in obtaining the data were of the mutant lordotic strain and were raised in the laboratory under controlled environmental conditions.

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Some few wild-type fishes, obtained from local aquarists, have been included in the results since no apparent differences could be found between the two strains by analysis of the individual data.

All fishes were maintained at a temperature of 72° F. (range 70°–75° F.) except during the summer months when observations were discontinued. The aquaria were illuminated by reflectors containing light bulbs for 10–12 hours daily, since natural lighting was absent in the aquarium room. The fishes were fed daily a varied diet of commercial dry foods with supplements of Tubifex worms at least twice weekly and fresh or frozen Daphnia when available.

Virgin female fish were obtained in the usual manner (Rosenthal and Rosenthal, 1950). They were used in the breeding studies when full maturity was reached at an age of 4–8 months. For study, all fishes were kept in individual numbered gallon aquaria containing sprays of *Ceraptopterus* during the entire period of observation. When transfers of fish from one aquarium to another were necessitated for maintenance or experimental procedures, tap water [2] was aged in the presence of *Ceraptopterus* and snails for at least a week prior to use. Water thus aged was never observed to cause any shock or toxic symptoms in fishes. Female fishes were mated with two males for twenty days. They were then isolated until parturition and re-mated with two males after a short rest of 24–48 hours. The progeny of each brood were counted shortly after birth. Embryos that never became fully activated were probably eaten by the female parent, but the evidence is good that most viable young escaped cannibalism. Mothers that had apparently aborted or eaten their young (a rare occurrence in well fed fish) were not considered in the data unless the birth was actually observed (Rosenthal, 1951b).

RESULTS

Early in the work, it was observed that the interval between the birth of successive broods, under our conditions of temperature and light, averaged 31.1 days (standard error = 0.4), with a range of 23 to 37 days, as shown in Figure 1. However, when virgin females were mated, the initial brood was born at any time from 23 to 45 days with no evidence of any regular sequence. The results of 40 observations are presented graphically in Figure 2. With the exception of one female, who bore her initial brood 45 days after mating, fishes failing to produce broods within 42 days were invariably sterile, although they were kept under observation for 90 days. The 42 day period, 6 days less than twice the gestation period, was short enough to eliminate the possibility of a mother aborting an unobserved first brood and presenting a second brood within the interval.

The wide spread in time for first broods is not due to delayed copulation as shown in Table I. These data were obtained by placing a virgin female *Lebistes* with two males for the requisite period of time. The ovaries were then removed, teased apart in a drop of 0.8% sodium chloride on a glass slide, and examined for motile sperm cells under a high power objective. Only one of five ovaries contained sperm after 12 hours, but six ovaries of seven contained sperm after 24 hours, and all ovaries contained sperm at the end of 48 hours. A definite mating reaction consisting of arching, jabs, and thrusts (Clark, Aronson and Gordon, 1948, 1949; Schlosberg, Duncan and Daitch, 1949) on the part of the male fishes was usually evident within 15 minutes after the addition of the female, and it is assumed that copulation occurred rapidly, although actual copulation was probably never observed (Clark and Aronson, 1951).

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[2] These studies were completed in New Brunswick, New Jersey, during the author's tenure at Rutgers University. The tap water obtainable in this city was remarkably free of permanent substances noxious to fishes. Although chlorinating agents were present in fresh tap water, ageing of the water in glass containers for 24 hours was found to be sufficient to remove these materials, and to make the water suitable for the culture of fishes.

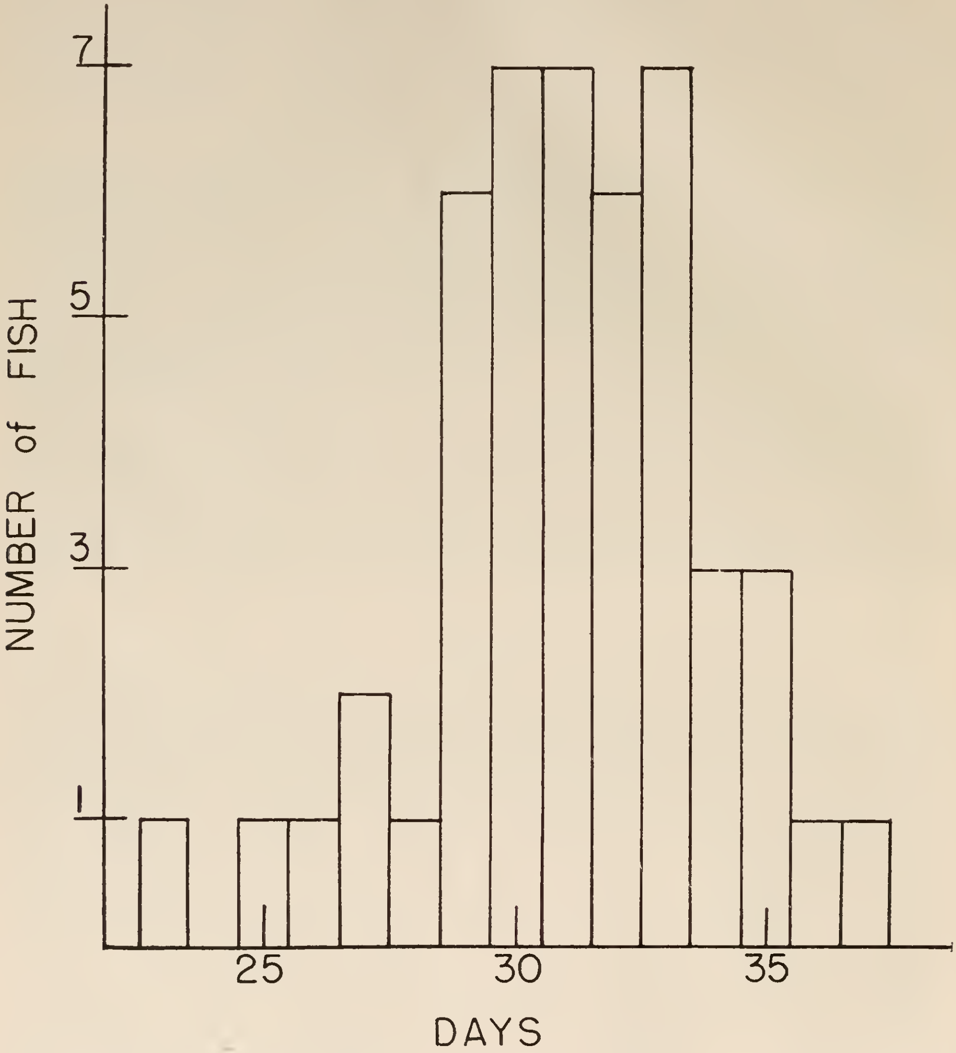

FIGURE 1. Frequency distribution of the interval between 47 successive broods for female *Lebistes*.

Table I

Time between mating of virgin female Lebistes and the presence of sperm in the ovary

Number of females	Hours after mating	Sperm in ovary	
		Present	Absent
5	12	1	4
7	24	6	1
6	48	6	0

The presence of active sperm in the ovary can be taken to mean that fertilization will occur as soon as a sperm cell meets a receptive ovum. To test this hypothesis, and also to yield information on the time of fertilization with respect to a previous brood, the lordotic phenotype, which is recessive to the wild-type (Rosenthal and Rosenthal, 1950), was used as a tracer. To carry out the experiment, virgin females of the lordotic strain were initially mated with two lordotic males. After 20 days the females were isolated to await delivery of the first brood, thus establishing the fertility of the female, and the presence of lordotic "tracer" sperm in the ovary. The progeny of the first mating were recessive mutants. At intervals following the birth of the brood, the females were re-mated with two wild-type males for 20 days. The progeny of the second brood must contain either all mutant or mixed mutant and wild-type young, depending on the sperm cell that brought about fertilization. The ratio of mutant : wild-type young was taken as a measure of sperm replacement and can be used to indicate (1) the time necessary for sperm to reach the ovary and fertilize the mature ova after mating, and (2) the time necessary for ova to reach a fertilizable stage relative to the delivery of the

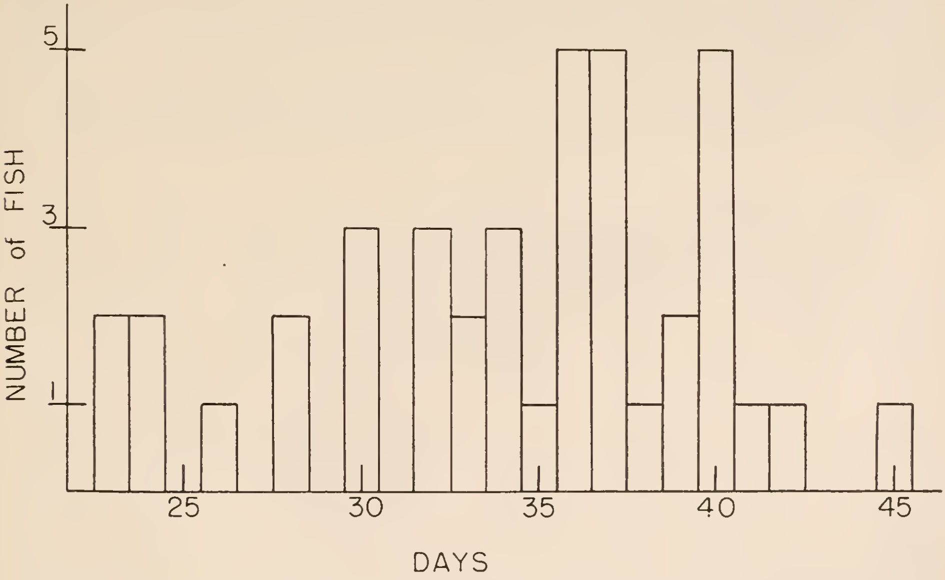

Figure 2. Frequency distribution of the interval between the time of mating of 40 virgin female *Lebistes* and the birth of the first brood.

TABLE II

The time of maturation and fertilization of ova in Lebistes reticulatus as determined by the use of mutant and wild-type fishes. See text

Days after brood delivery	Number of females	Sperm replacement*	Ratio of mutant: wild-type progeny	Average per cent replacement
1	3	x, x, x	10/50	83
4	4	x, x, x, x	14/21	60
6	5	x, x, x, 0, 0	36/20	36
8	4	0, 0, 0, 0	36/0	0

* x indicates sperm replacement; 0 indicates no sperm replacement.

first brood. The results obtained by this procedure are shown in Table II. It is apparent that sperm replacement occurred when the mothers were re-mated between one and four days following the first brood. If 8 days elapsed before re-mating, no placement occurred. However, when mothers were re-mated on the sixth day postpartum, three of five females showed sperm replacement but two did not, indicating a border-line condition when the ova were still unfertilized by retained sperm of the first mating. These data demonstrate conclusively that copulation, sperm migration through the gonaduct of the female and amphimixis occur rapidly after mating. Assuming an interval of at least 24 hours between copulation and fertilization, the time of maturation of ova in *Lebistes* is approximately 5 days. This 5 day period is shorter than the 7 day interval for the maturation of ova, as determined by cytological methods for fishes reared at comparable temperatures (Hopper, 1943; Tavolga and Rugh, 1947; Turner, 1937).

It is evident, since the gestation period is approximately 25 days in length, that the ova of virgin female *Lebistes* vary greatly in the degree of receptivity to fertilization. An inspection of the ovaries of 47 freshly killed virgin females showed that all but two contained large, apparently fertile orange-yellow ova about 1.5 mm. in diameter. The two exceptions mentioned contained degenerate ova about 0.2 mm.

TABLE III

Brood record of seven individual female Lebistes illustrating maximum and minimum periods of brood production

Brood	Fish's number						
	1	3	5	21	24	25	26
1	4	4	3	2	16	16	12
2	5	4	4	19	32*	33*	34*
3	15*	4	4	31*	39*	2	25
4	8	5	19*	7	19	20*	10
5	16*	9*	4	4	20	18	20
6	4	3	36*	—	—	4	—
7	5	—	—	—	—	—	—
8	1	—	—	—	—	—	—

* Indicates apparent maxima.

in diameter that were chalky white in color, and the fishes were probably not fertile. Occasionally, some of the ovaries contained a second group of small ova about 0.7–0.9 mm. in diameter. The two groups of ova of different size may represent superfoetation (a condition rare in *Lebistes* and never observed during this study) or a cyclical development and resorption of unfertilized ova analogous to an estrus cycle in higher vertebrates. Attempts to elucidate further this hypothesis by studying the cellular changes in the oviduct with the smear technique of Clark (1950) have not been successful, due to traumatic changes that occurred and to the lack of desquamated cellular elements which could be obtained by this method.

The brood record of seven females is shown in Table III. In general, initial broods are small in number, depending largely on the size of the female (Turner, 1937). Although exact measurements were not made, the fishes were all approximately equal in size when they were first mated. It is apparent that the number of young per brood increases to a maximum, then decreases to a minimum. If a sufficient number of broods is obtained from individual females, the maxima and minima may again be evident. Females 1, 5, and 25 illustrate two such cycles of large and small broods for eight, six and six consecutive intervals, respectively. Females 3, 21, 24, and 26 show variable but definite reactions toward cyclical brood production. The data resemble the seasonal effects of light in stimulating the reproductive activity of fishes (Aronson, 1951; Hoover and Hubbard, 1937; Rowan, 1926; Turner, 1937). This fluctuation of light with the seasons may be important under natural conditions but not under the controlled conditions of the present experiments.

TABLE IV

Average number of mature ova in virgin Lebistes, and the number of young per brood in first and subsequent broods

	Virgin ovaries	First brood	Subsequent* broods	All broods	Maximum† brood
Number of fish	27	40	63	103	21
Average brood size	9.2	9.1	13.3	11.7	23.8
Range	3–25	2–29	2–39	2–39	15–39
Standard error $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$	±1.2	±1.1	±1.3	±1.5	±1.7

* The values do not include the first brood.
† Derived from mature females having 3 or more broods.

The data illustrated in Table IV show that the number of ova present in virgin fishes and the number of young born in the first brood are the same. The average number of young in all broods (11.7) is somewhat smaller than the average of all broods (13.3) when the initial brood is omitted from the calculations. An average maximum brood (23.8) is shown for 21 females who had a record of at least three broods. Although a single female has been recorded to deliver 71 fry in one brood (Purser, 1938), the largest brood observed over a two-year period in this investigation was 39, a value consistent with the reports in popular aquarium literature.

DISCUSSION

The present investigation has revealed striking differences in reproduction of *Lebistes* as compared with earlier work. The wide spread in time for production of first broods suggests an ill-defined estrus cycle in which ova mature and, in the absence of fertilization, are resorbed periodically. Degeneration of ova in unfertilized females has been demonstrated for *Heterandria* (Fraser and Renton, 1940), *Lebistes* (Liu, 1937), *Gambusia* (Thirumallacher: as quoted by Fraser and Renton, 1940) and the Goodeidae (Turner, 1933). These reports indicate that the process of resorption in these different live-bearing cyprinodonts are similar. However, no reports have appeared in the literature to elucidate more completely the length of time that mature ova retain their fertility prior to degeneration, or to the relative maturation time of younger ova to those undergoing degeneration.

Although an estrus cycle has been proposed in *Lebistes* by Jaski (1939), based on a cyclical change of angle at which the female swims, this report has not been substantiated and is thoroughly discussed by Clark and Aronson (1951). However, Jaski (1939) also reported that virgin females did not come into estrus for three to four days after they were introduced to aquaria containing males, and mating was practically impossible for several days due to the non-receptivity of the females. The observations reported in Table I indicate conclusively that copulation occurs within 24 hours, although fertilization need not necessarily follow as shown by the data in Figure 1. On the other hand, mature ova are rapidly fertilized by fresh sperm as shown in Table II.

Clark and Aronson (1951) have suggested that some females are at times more sexually receptive than others. The degree of receptivity is difficult to measure, but observations made during the past three years tend to substantiate these reports. In general, virgin females who have been maintained in aquaria free of male fishes of any sort tend to pursue male fishes when they are placed together. Receptivity in the female also increases shortly after the birth of a brood, or rather, male fishes tend to pursue more vigorously females that have just delivered young. Whether increased sexual activity on the part of the male is due to a release of sexually stimulating substances by the female, or whether the female exerts stimulatory activity by her actions on the male is a question which requires further clarification.

Female fishes of the family Poeciliidae are unique among vertebrates in their ability to retain active sperm in ovarian tissue for long periods of time and to bear successive broods of young without re-impregnation. Thus, Van Oordt (1928) has reported eight successive broods for a female *Xiphophorus* after a single insemination. Winge (1922, 1937) has also reported the succession of broods in the absence of male fishes for eight months in *Lebistes*. Under these conditions, the size of subsequent broods tends to decrease or brood production may cease entirely, especially during the winter months (Breder and Coates, 1932; Breider, 1934; Van Oordt, 1928). On the other hand, these investigators have reported that females constantly re-inseminated continue to produce broods of large size.

The data presented in Table III suggest that the peaks of sexual activity appear to deplete the animals of anterior pituitary gonadotrophins or may bring about a refractory period due to metabolic exhaustion, as suggested by Bissonette (1937) and Miller (1948) in their studies with fishes and birds. These data cannot be compared with previous work, since in earlier reports light conditions were not controlled, and for the most part, breeding females were able to rest during the winter months. Turner (1937) has indicated that brood production in poeciliids under natural conditions reaches a maximum during late spring and early summer and decreases to a minimum during the fall and winter months.

The size of a brood produced by female poeciliids under a natural or simulated environment has been shown to be correlated with the size of the mother, large fishes usually giving birth to broods of large size (Purser, 1938; Turner, 1937). The data shown in Table III and IV indicate that factors other than the size of the female play an important role in brood production. In general, the number of young produced in a brood is largely dependent on the physiological condition of the mother. The factors affecting brood production must therefore await further clarification.

SUMMARY

1. Evidence is presented to indicate an as yet ill-defined cyclical production of ova in virgin female *Lebistes*.

2. Copulation occurs within 12–24 hours after virgin female fishes are placed in aquaria with two male fishes. Sperm migration in the female gonaduct and amphimixis occurs rapidly after mating.

3. The time of fertilization of ova in relation to a previous brood has been determined by the use of sperm replacement studies with genetic mutation tracers.

4. Evidence is presented to indicate a cyclical nature of maximum and minimum brood production of individual female fish maintained under constant conditions of temperature and light.

5. The possible factors affecting brood production are discussed.

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TYROSINASE AND THE RESPIRATION OF PUPAE OF PLATYSAMIA CECROPIA L.[1, 2]

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It has been suggested that the enzyme tyrosinase functions as a terminal oxidase in the respiration of insects and other animals (Bhagvat and Richter, 1938; Heller, 1947; Sussman, 1949). Although there has been little experimental evidence from work done on insects to support this view, an abundance of such data is available from investigations conducted with plant materials. For example, Boswell and Whiting (1938, 1940), Boswell (1945), Baker and Nelson (1943), Bonner and Wildman (1946), and others have claimed that tyrosinase functions as part of a respiratory shuttle system in the manner shown in Figure 1 (Nelson, 1950).

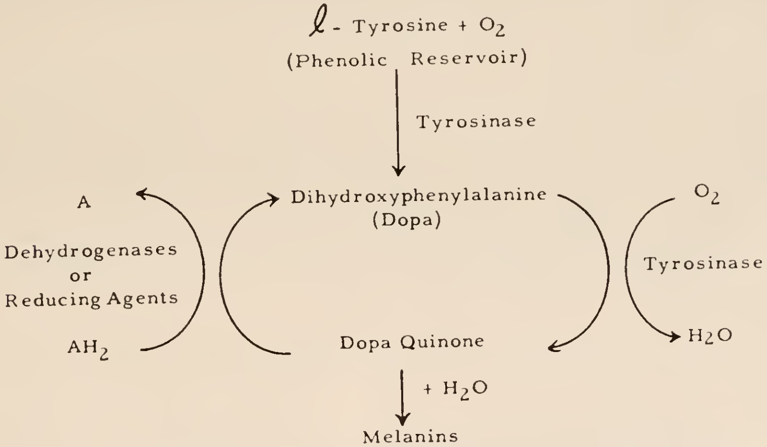

FIGURE 1. A possible mechanism for the participation of tyrosinase in respiration. (Modified from Dawson and Tarpley, 1951.)

In this system, dopa serves as the hydrogen carrier adjacent to the terminal oxidase, while tyrosine acts as a reservoir to supply dopa to the system. Moreover, if for some reason this respiratory cycle is disturbed and the reduction of the quinone is prevented, the shuttle breaks down and melanins are formed. Indeed,

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[2] The author would like to express his gratitude to Dr. William H. Weston for his sympathetic and stimulating direction of this work. I should also like to state my appreciation for the cooperation and help given by Dr. Kenneth V. Thimann and Dr. Carroll M. Williams during the course of these investigations. Finally, I should like to thank Dr. David R. Goddard for his penetrating and effective criticism of this manuscript.

such "decompensated respiration" has been claimed to occur in the case of certain fungous diseases of plants (White, 1945). On the other hand, some recent evidence suggests that the role of tyrosinase in plant respiration may be more limited than has hitherto been thought (Levy, Schade, Bergmann and Harris, 1948; Goddard and Holden, 1950).

The almost universal presence of tyrosinase in insects, coupled with the blackening of the blood of these insects due to trauma or disease (Sussman, 1951) suggested that a respiratory mechanism, paralleling that postulated above for plants, is in operation in these animals. Consequently, in order to investigate the possibility that tyrosinase is used as a terminal oxidase in insect respiration, and that "decompensated respiration" occurs as a result of disease in these animals, the writer undertook the following experiments.

Materials and Methods

The animals used in these experiments were diapausing pupae of *Platysamia cecropia* obtained from Dr. C. M. Williams.

Respirometric experiments were conducted with both intact pupae and with tissue homogenates prepared from these. The respiration of the intact animal was measured by the use of Scholander-Edwards microrespirometers (Scholander, 1942; Scholander and Edwards, 1942), which were immersed in a constant temperature water bath kept at 24° C. ± .5° C. The instruments were suspended in water-filled plastic containers which rested on a support on the bottom of the water bath. In order to remove CO_2 during the measurements of the Q_{O_2}, about 100 mg. of soda lime (ascarite, 8–20 mesh) or 1 cc. of 5% KOH were placed at the bottom of the shell vial containing the animal. Equilibration was accomplished by allowing the instrument to remain open to air for 15 minutes, after which oxygen was added and the system was closed to air. In order to allow the oxygen to come to the temperature of the water bath, seven more minutes were allowed to elapse before the first reading was made.

Injection of the various substances to be tested for their effect on the oxygen uptake of the intact animal was accomplished by use of the techniques outlined in a previous paper (Sussman, 1951).

The oxygen uptake of tissue homogenates was measured by the use of Warburg respirometers which were maintained at a temperature of 24° C. and shaken at a rate of 130 oscillations per minute. The reaction vessels contained 2.0 cc. of the tissue homogenate and 0.2 cc. of 10% NaOH, while the sidearms contained 0.3 cc. of a solution of various substances to be tested for their effect upon the respiration of the tissues. Tissue homogenates were prepared by cutting pupae into sections with a pair of scissors and placing the cut pieces in the bowl of a Waring blendor. To this was added 9 parts (by weight) of a 0.1 *M* phosphate buffer (pH 6.8), after which the mixture was minced in the blendor for 5 minutes. The resulting "brei" was then poured into a glass homogenizer tube and was ground for 5 minutes with a motor-driven glass pestle. By this procedure a thoroughly blended preparation was obtained which could then be pipetted into Warburg flasks. All operations prior to the actual respiratory measurements were carried out in the cold.

Results

Experiments with intact pupae

The respiration of the intact animal was studied by determining the effect of the injection of the following kinds of compounds:

1. Phenolic substrates for tyrosinase
2. Inhibitors of tyrosinase

Before the effect of the injection of these substances could be measured, it was necessary to learn to what extent the injection technique itself would affect the animal's respiration. With this in mind, varying amounts of distilled water and of insect Ringer's solution were introduced into the animal, after which its oxygen uptake was measured. Figure 2 and Table I show the results of the injection of .05 cc. distilled water and .1 cc. of insect Ringer's solution into the animal. It is apparent from these results that a rise in Q_{O_2} occurs upon the injection into the animal of either distilled water or insect Ringer's solution. This increase was found to occur whenever the integument of the animal was broken upon the insertion of the syringe needle, even if nothing was injected, so that it was necessary to include this factor in calculating the results of the subsequent injection experiments. That this increase in respiration was not due merely to increased movements of the

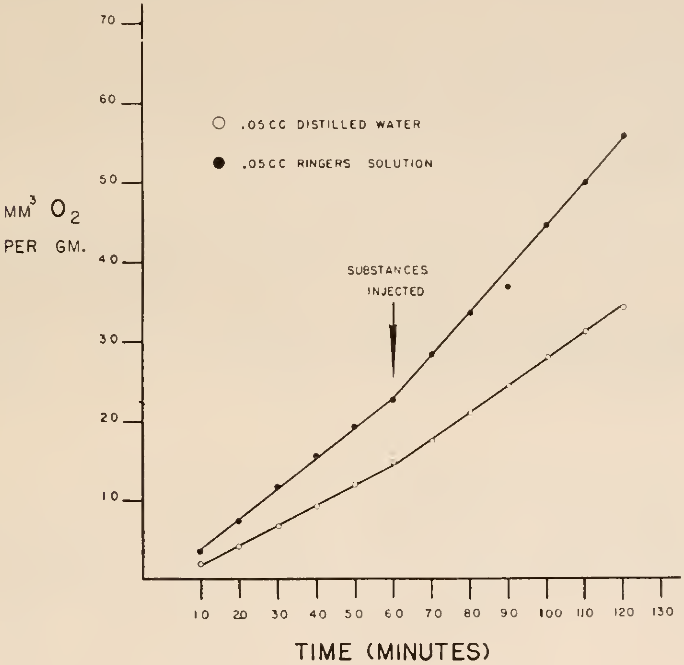

Figure 2. Effect of injection of distilled water and Ringer's solution on the oxygen uptake of pupae of *P. cecropia.*

TABLE I

Effect of injection of 0.05 cc. distilled water or insect Ringer's solution on Q_{O_2} of intact diapausing pupae of P. cecropia

Treatment	Q_{O_2}	Standard deviation	No. of samples
None	0.015	±0.0026	37
0.05 cc. dist. H_2O or Ringer's solution	0.022	±0.0081	11

animal is shown by observations over the period of a week, which indicate that the animal retains the increase in Q_{O_2} for this period.

Having determined the effect on the pupae of the injection of water, it was now possible to investigate the effect of the substrates and inhibitors which were injected dissolved in water into the animals.

Tyrosine and protocatechuic acid were chosen as substrates since they have been claimed to function in the respiratory cycle of certain plants (Baker and Nelson, 1943), and are also known to be present in insects (Pryor *et al.*, 1946; Wigglesworth, 1950). Similarly, adrenalin was used because one of its oxidation products, adrenachrome, was shown to function as a hydrogen carrier in certain animal polyphenolase systems (Bhagvat and Richter, 1938; Dawson and Mallette, 1945). Although catechol has not been found to occur naturally in the tyrosinase systems which have been studied, it has been used in the study of potato respiration by Boswell and Whiting (1940), so that it was included in these studies.

The results of the injection of these phenolic compounds, as compared with those obtained upon the injection of distilled water and insect Ringer controls, are given in Table II.

It can be seen from Table II that none of the substances injected, except 10^{-1} M catechol, produced a significant rise in the Q_{O_2} of the intact animal. That the increase due to high concentrations of catechol was a toxic response is suggested by

TABLE II

Effect of the injection of various phenolic substrates upon the Q_{O_2} of intact diapausing pupae of P. cecropia

Compound	Concentration	Amount injected	% Increase in Q_{O_2}	Significance of increase in Q_{O_2} compared with that obtained for water*
Distilled water	—	.05 cc.	33	none
Insect Ringer's	—	.1	44	none
Catechol	10^{-2} M	.05	50	none
Catechol	10^{-1} M	.05	156	+
Catechol	1 M sat'd sol'n	.05	—**	No reading possible
Tyrosine	(.04%)—10^{-3} M	.05	15	none
Adrenaline	1:1000	.05	43	none
Protocatechuic acid	10^{-2} M	.05	19	none
Protocatechuic acid	10^{-1} M	.05	37	none

* The significance of these results was calculated according to the group comparison tests given in Chapter 4 of Snedecor (1946).

** Immediately upon the injection of .05 cc. of 1 M catechol, all four of the animals tested exuded a yellow fluid which appeared to consist of blood and epithelial cells. Such exudation continued for some time after which the blood blackened and the animals died.

the fact that the animals became very irritable and actively moved their abdomens upon the injection of 10^{-1} *M* concentrations of this substance. Furthermore, a more definite toxic response was elicited by the introduction of 1 *M* catechol since the animals exuded blood and usually died soon after the injection of the chemical. These reactions were not elicited upon the injection of lower concentrations of catechol and all concentrations of the other substances tested.

The inhibitors used were chosen for their potency and for their solubility in water. Table III gives the effects of the injection of these compounds upon the oxygen uptake of pupae of *P. cecropia.*

These results indicate that tyrosinase inhibitors, far from depressing the Q_{O_2}, actually increased this figure in certain cases. In no instance was the oxygen uptake significantly inhibited, as would be expected if tyrosinase were involved in respiratory processes. Similarly, the injection of iodoacetic acid and malachite green failed to induce any significant response.

TABLE III

Effect of the injection of tyrosinase inhibitors upon the respiration of intact diapausing pupae of P. cecropia

Compound	Concentration	Amount injected	% Increase in Q_{O_2}	Significance of increase compared with that obtained upon injection of dist. H_2O
Sodium Diethyldithiocarbamate	10^{-2} *M*	.05 cc.	19	none
Sodium Diethyldithiocarbamate	10^{-1} *M*	.05 cc.	67	none
Sodium Diethyldithiocarbamate	1 *M*	.05 cc.	57	none
Salicyl Aldoxime	10^{-2} *M*	.05 cc.	31	none
Salicyl Aldoxime	10^{-1} *M*	.05 cc.	130	+
Iodoacetic Acid	10^{-2} *M*	.05 cc.	38	none
Malachite Green	120 parts/ million	.05 cc.	25	none

In order to overcome the objection that the concentrations of the compounds used as substrates and inhibitors of tyrosinase were not physiological, dilutions as low as 10^{-5} *M*, of all the substances injected previously, were tested as above. The results showed no more effect than did the injection of water so that it was plain that the concentrations used made no difference in the results.

Experiments with tissue homogenates

The oxygen uptake of tissue homogenates prepared from the intact animals was next investigated. To the homogenates, prepared as described previously, were added certain phenolic substrates whose effect upon the oxygen uptake of the tissue was to be determined. The results are given in Table IV. As in the case of the intact animal, catechol produced the greatest increase in oxygen uptake. However, it should be noted that this rise was only transient since the Q_{O_2} fell rapidly within the period during which the readings were taken. Moreover, the homogenate turned reddish brown within an hour after the catechol solution was introduced. Tyrosine increased the Q_{O_2} of the homogenate, but to a much smaller extent than did the catechol. The homogenate became greyish black an hour after the introduction of the tyrosine. Protocatechuic acid was the only one of the substrates added which failed to produce a rise in the oxygen uptake. In fact, in these experiments an actual decrease in Q_{O_2} was noted when this compound was added and no color change was noted. This is indeed curious since most tyrosinase preparations from other sources usually oxidize this compound.

In order to determine the effect of inhibitors upon the Q_{O_2} of the homogenate, malachite green, a dehydrogenase poison (Takahashi, 1948; Caldwell and Meikeljohn, 1937; Quastel and Wheatley, 1931), and iodoacetic acid, a sulfhydryl poison (Olcott and Fraenkel-Conrat, 1947), were used. Six parts per million of malachite green and 10^{-3} M iodoacetic acid were added to the homogenate before it was placed in the Warburg vessels, and the Q_{O_2} determined as before. The results showed that malachite green and iodoacetic acid inhibited the oxygen uptake of homogenates 36% and 60%, respectively. Therefore, ⅔ to ½ of the Q_{O_2} of the homogenate is dependent upon the action of enzymes which are poisoned by malachite green and iodoacetic acid.

TABLE IV

Effect of addition of various phenolic compounds on the Q_{O_2} of tissue homogenates prepared from pupae of P. cecropia

Compound added	Final concent. in homogenate	% Increase in Q_{O_2}		
		Time after addition of compounds		
		15 Min.	30 Min.	45 Min.
Catechol	6×10^{-2} M	2800	2200	2000
Tyrosine	3×10^{-4} M	592	368	274
Protocatechuic acid	6×10^{-2} M	0	0	0

It was then attempted to determine whether the presence of inhibitors would interfere with the oxidation of phenolic substrates added to the homogenates. The inhibitors were added as in the preceding experiments and the Q_{O_2} of the homogenate was determined before and after the introduction of the substrate, tyrosine. From the results it became apparent that the increase in Q_{O_2} attendant upon the addition of tyrosine is not affected by the presence of malachite green since only 5% inhibition could be demonstrated. On the other hand, this increase is inhibited 37% by the addition of iodoacetic acid.

DISCUSSION AND CONCLUSIONS

On the basis of the foregoing data, it must be concluded that tyrosinase does not function as a terminal oxidase in the respiration of the intact diapausing pupa of *P. cecropia*. This is demonstrated by the fact that the addition of phenolic substrates did not serve to increase the Q_{O_2} of the animals significantly, while the addition of tyrosinase inhibitors failed to depress this figure. Another line of evidence which lends support to this data is that derived from some unpublished experiments of Dr. C. M. Williams, who has found that even crystals of phenylthiourea and sodium diethyldithiocarbamate placed directly in the body cavity of diapausing pupae fail to prevent development of the adult.

It may, perhaps, be argued that the various compounds injected were not used in physiological concentrations. This objection is largely overcome by the results of the experiments using concentrations of the substrates and inhibitors as low as 10^{-5} *M* which showed essentially the same effects as those in which higher concentrations were used. On the other hand, these data do not definitely exclude the possibility that the concentrations of inhibitors used were not sufficient to overcome possible "protective" effects exerted by certain metabolites on enzyme systems, examples of which have been shown to occur in the case of succinic dehydrogenase (Hopkins, Morgan and Lutwak-Mann, 1938) and oat coleoptiles (Thimann and Bonner, 1948). However, the stimulating effect of 10^{-1} *M* salicyl aldoxime on the animal's oxygen uptake suggests that this concentration of the inhibitor is affecting one or more of the enzymes concerned with respiration so that it is possible that the protective effect is overcome at this concentration. That tyrosinase is not one of these enzymes is proven by the fact that the effect of the inhibitor is one of stimulation, rather than inhibition which would be expected to occur if a copper protein were involved in respiration.

TABLE V

Differences between the enzyme systems involved in the oxygen uptake of intact diapausing pupae of P. cecropia and the tissue homogenates made from them

Intact diapausing pupa	Tissue homogenate
1. $Q_{O_2} = .015 \pm .0026$	1. $Q_{O_2} = 1.0 \pm .18$
2. Insensitive to copper protein poisons	2. Sensitive to copper protein poisons
3. Insensitive to sulfhydryl poisons (iodoacetic acid)	3. Oxygen uptake reduced 60% by addition of 1×10^{-3} *M* iodoacetic acid
4. Insensitive to addition of phenolic substrates for tyrosinase in concentrations below 1×10^{-1} *M*	4. Oxygen uptake increased upon addition of 3×10^{-4} *M* tyrosine, and 6×10^{-2} *M* catechol

Mention should also be made of the fact that catechol, in a concentration of 1×10^{-1} *M*, produced a sharp rise in the oxygen uptake of the intact animal. Although this effect seems to suggest that this compound is being used as a respiratory substrate, it is felt that the high concentrations required to achieve this effect argue against this interpretation. Instead, it would seem that the effect noted is a toxic effect of the chemical which disrupts the animal's respiration. Such a toxic effect of catechol upon respiratory processes was noted by Boswell and Whiting (1940) and others, who finally had to use more physiological substrates like protocatechuic acid in their experiments on potato tyrosinase. Further evidence to support this contention is supplied by the fact that injection of the next higher concentration of catechol used in these experiments (1 *M*) served to blacken and kill the animal. In the face of this evidence, then, it is hard to believe that tyrosinase plays any significant role in the respiration of the diapausing pupa.

On the other hand, tyrosinase does seem to be responsible for part, at least, of the oxygen uptake of tissue homogenates prepared from diapausing pupae. The enormous increase in Q_{O_2} which resulted from the addition of phenolic substrates would argue for this interpretation.

In comparing the systems responsible for the oxygen uptake of the intact diapausing pupae and the tissue homogenates prepared from these, significant differences are at once apparent. These differences are summarized in Table V. It should be noted that the conclusions on the sensitivity of homogenates to copper protein poisons were taken from unpublished experiments of Dr. Richard Sanborn.

In the light of these results, it is of interest to analyze the data obtained by Heller (1947) in his work on tissue homogenates of *Celerio euphorbiae* pupae. This author claimed, on the basis of his experiments with homogenates, that tyrosinase functions as a terminal oxidase in the respiration of pupae of *Celerio*. He found that the oxygen uptake of pupal homogenates increased and blackening was retarded upon the addition of a cozymase preparation or a boiled yeast extract. Heller's inference that cozymase is the main H_2 acceptor from metabolites and that the tyrosinase system is concerned with further transport and oxidation of the hydrogen is probably justified, since he showed a prolongation of respiratory activity as a result of the addition of cozymase. However, he failed to consider the fact that tyrosine is present in yeast preparations, so that the rise in respiration may have been partly due to the addition of substrate for the action of tyrosinase. In general, however, Heller's results seem to agree with those of the author insofar as it can be concluded that tyrosinase functions as a terminal oxidase in the oxygen uptake of tissue homogenates of pupae. On the other hand, there is considerably less agreement when Heller extrapolates from the situation in his "brei" preparations to that in the intact pupa and states that his results establish the role of tyrosinase in insect metamorphosis as a terminal oxidase in the respiration of the pupae. It is felt that the writer's researches have demonstrated that very fundamental differences exist between the respiratory systems of the intact animal and those of the homogenate, and that these differences are of such a nature as to preclude the possibility that tyrosinase functions in the respiration of the diapausing pupa of *Platysamia cecropia*. However, it is still open to question whether or not a respiratory role can be assigned to tyrosinase in other stages of the insect's development.

SUMMARY

1. The Q_{O_2} of diapausing pupae of *Platysamia cecropia* is not affected by injection of substrates or inhibitors of the enzyme tyrosinase.

2. The Q_{O_2} of homogenates prepared from such animals is markedly influenced by the above reagents.

3. On the basis of this and other data, it is concluded that tyrosinase does not function as a terminal oxidase in the respiration of the diapausing pupa.

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THE MECHANISM OF ACTION OF ACETYLCHOLINE ON THE VENUS HEART[1]

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It has long been known that faradic stimulation of the visceral ganglion of the bivalve mollusc *Venus mercenaria* produces an inhibition of the heart (Budington, 1904; Carlson, 1905). Prosser (1940) showed that the cardio-inhibitory mechanism probably involved the release of acetylcholine at nerve endings. Recent study of the action on the Venus heart of cholinergic drugs and acetylcholine-blocking agents has shown the inhibitory action of acetylcholine to be nicotine-like and readily blocked by tetraethylammonium ions (Welsh and Taub, 1948; 1950).

The effects of direct electrical stimulation of isolated molluscan hearts have also been studied, and it has been shown that anodal current applied to the heart results in a depression of the heart beat (Arvanitaki and Cardot, 1933; Arvanitaki, 1938; Jullien and Marduel, 1938a; 1938b). In the light of the similarity of these two responses, the molluscan heart would seem to be a suitable object for the determination of the relationship, if any, between inhibition by direct current and by acetylcholine.

The investigation of such a relationship involves questions such as: (1) does acetylcholine affect a fundamentally electrical process such as resting potential of the muscle fibers, or ion distribution, thereby producing its characteristic inhibitory effect, and (2) does appropriate electrical stimulation affect the same electrical mechanism or does it merely influence one of a complex series of events only indirectly concerned with contraction and acetylcholine metabolism?

The approaches we have used are: (1) the study of electrical manifestations produced by treatment of the heart with acetylcholine and with a blocking agent by following changes in the electrocardiogram; (2) the investigation of chemical responses of the heart to electrical stimulation by perfusion of internal fluid from the stimulated heart to a second heart; and (3) the study of the effects of an acetylcholine-blocking agent on the responses to electrical stimulation.

MATERIALS AND METHODS

A. Electrical stimulation

Isolated hearts from large specimens of the quahog, *Venus mercenaria L.*, collected from Narragansett Bay were used. The apparatus (Fig. 1) for the stimulation of the heart was essentially similar to that used by Arvanitaki (1938). The

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heart was suspended in sea water between a thread tied around one atrioventricular junction leading to a light heart lever and a cannula (C) inserted into the opposite atrioventricular opening. The two aortae were ligated. The stimulating electrodes, S_1 and S_2, were silver-silver chloride electrodes, one of which (S_2) was dipped into the sea water filling the cannula, and the other electrode (S_1) was a ring of heavy wire immersed in the bath and surrounding the heart. Because of the high resistance due to the small pore-size of the cannula, the ring electrode was considered to be the "effective" electrode, and the cannula electrode the "indifferent" electrode. Thus, when the ring electrode was negative, the stimulation was considered to be "cathodal stimulation" and its effect on the heart as "catelectrotonus," and when positive as "anodal stimulation" and "anelectrotonus." The stimuli were provided by a Grass Model 3-C stimulator equipped to give either repetitive or DC stimuli.

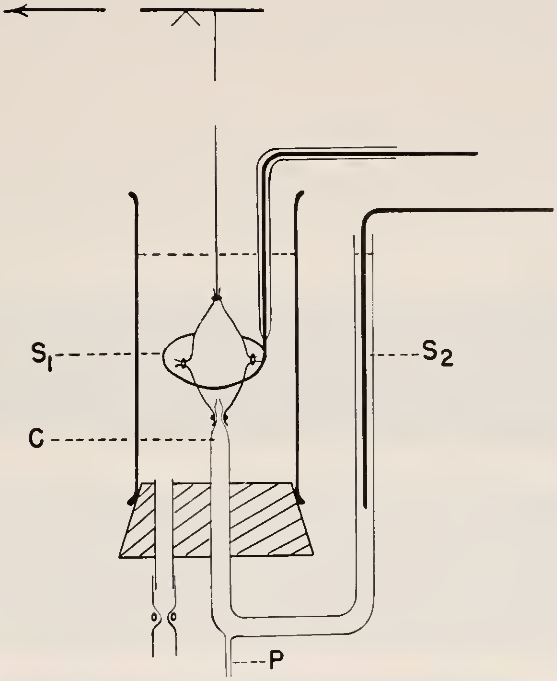

FIGURE 1. Heart bath with electrodes arranged for stimulation. See text for further explanation.

B. Series perfusion

A modification of the arrangement in Figure 1 was used, in which a capillary tube led off from the bottom of the cannula at "P" to another cannula inserted into a receptor heart mounted in a separate chamber below. This isolated the receptor heart from the electrical field of the stimulated heart while keeping the volume of the perfusion fluid passing from donor to receptor heart to a minimum. The rate of perfusion was determined by counting the drops overflowing from the lower chamber. A small glass cannula was inserted into the upper atrioventricular opening of the stimulated heart to allow the heart to receive sea water from the bath.

C. *Electrical recording*

An arrangement similar to that of Figure 1 was also used to record the changes in electrical potential, or the electrocardiogram (ECG). The sea water was replaced with pure, light, mineral oil, and the ring electrode was replaced by a fine silver-silver chloride wire attached to the writing lever and hooked into a loop of the upper atrioventricular ligature, which had previously been soaked in sea water. In order to reduce the electrical resistance of the cannula electrode, a silver wire was led through the cannula pore "C," and heated to form a small ball tip which was then chlorided.

The electrodes led to a Grass P-3 pre-amplifier, then through a 4 μfd condenser into the DC amplifier of a Dumont type 304-H oscillograph. This arrangement gave a time constant long enough so that the relatively slow ECG could be recorded effectively. Tracings were photographed with a 35 mm. camera, while simultaneous kymographic records of the myogram were made.

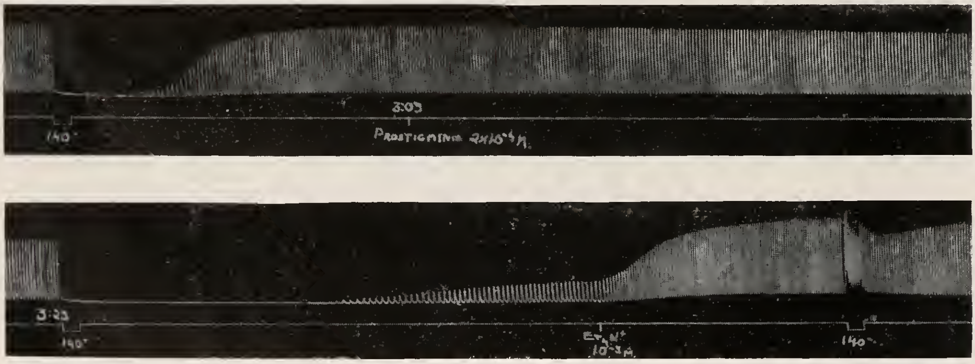

FIGURE 2. Record of the response of the isolated heart to a 30-second train of impulses before and after the addition of 2×10^{-4} molar prostigmine and 10^{-3} molar tetraethylammonium bromide (Et_4N^+).

.RESULTS

A. *Repetitive stimulation*

When the heart was stimulated with a train of impulses at 10 per second for 30 seconds, as in Figure 2, an inhibition similar to that produced by acetylcholine was observed. The addition of the anti-cholinesterases prostigmine or physostigmine greatly prolonged the inhibition produced by a second train of impulses at the same voltage, duration, and frequency as the first train. The addition of tetraethylammonium bromide (hereafter referred to as Et_4N) increased the rate of recovery. A third train of impulses similar to the first two produced very little inhibition when this acetylcholine-blocking agent was present in the bath.

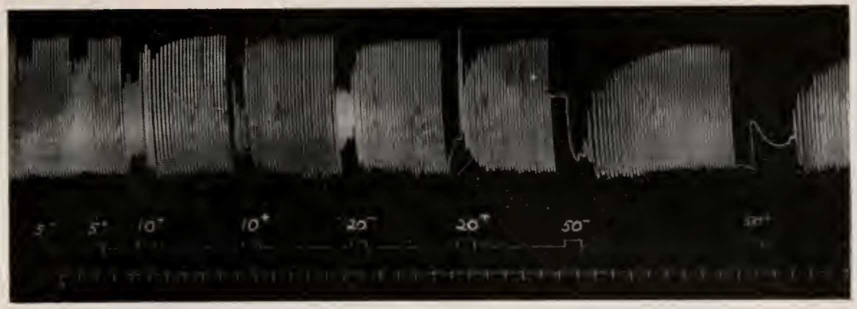

FIGURE 3. Responses of the heart to 30-second cathodal and anodal stimuli of various voltages.

B. Direct current stimulation

The responses of the Venus heart to constant currents are similar to those of the Helix heart as reported by Arvanitaki (1938) and Arvanitaki and Cardot (1933), and those of the heart of Mytilus observed by Jullien and Marduel (1938a). Figure 3 shows the responses to 30 second cathodal and anodal stimuli of various voltages. It can be seen that the cathodal stimuli increase the frequency of the beat and the tonus of the heart, while anodal current decreases the frequency and at higher voltages decreases the tonus. Lastly, while both anodal and cathodal stimuli decrease the amplitude, at any given voltage anodal stimulation is more effective.

Following strong DC currents, there is a brief reaction which resembles the response to current of the opposite polarity. This is also noticeable in Figure 7a and 7b where, after anelectrotonus, there is a strong contraction followed by a brief period of increased frequency, and after catelectrotonus there is an immediate relaxation followed by a brief period of decreased frequency. These responses are reminiscent of the post-cathodal and post-anodal overshooting of vertebrate nerve following electrotonus, reported by Lorenté de Nò (1947) and will be referred to as post-cathodal and post-anodal responses. These results suggested that the

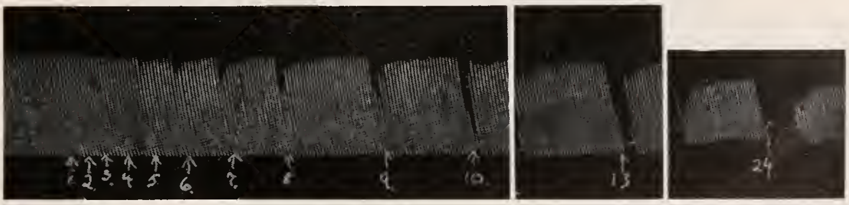

FIGURE 4. Responses of the heart to single anodal stimuli of durations from 0.02 milliseconds to 1 second.

1 = 0.02 msec.
2 = 0.05 msec.
3 = 0.1 msec.
4 = 0.2 msec.
5 = 0.5 msec.
6 = 1.0 msec.
7 = 2.0 msec.
8 = 5.0 msec.
9 = 10.0 msec.
10 = 20.0 msec.
13 = 0.15 sec.
24 = 1.0 sec.

effects of DC stimulation were not the result of nerve stimulation, but were the result of direct stimulation of the muscle tissue.

As a further check on the possibility that the inhibition is the result of stimulation of nerve endings, single anodal stimuli of 140 volts were given, the durations of which were varied stepwise from 0.02 milliseconds to 1 second. The responses of the heart (Fig. 4) were directly proportional to the durations. This supported the hypothesis that the effects were principally direct electrotonic effects on the muscle tissue, and suggested a direct experiment on the chemical response of the tissue to electrotonus using a series perfusion from a stimulated donor to a recipient heart.

C. Series perfusion

Of more than twenty attempts to record from the series perfusion arrangement, in only one instance were simultaneous records of the beat of the donor and receptor hearts obtained. The difficulty was principally due to the blocking of the cannula in the donor heart by the loosely arranged muscle fibers which tend to occlude the cavity of the heart. Figure 5 is a record of this experiment. The upper tracing is that of the donor heart, and the lower that of the receptor heart. The stimuli which were repetitive are labeled S_1, S_3, S_4, and S_5; while S_2 was a spontaneous inhibition. The responses of the receptor heart are labeled R_1, R_2, etc., with subscripts corresponding to the stimuli, the responses to which probably gave rise to these reactions. The response consists of an acceleration, a sudden deceleration, and recovery. The last three responses of the receptor heart, timed at the point of sudden change, follow the electrical stimuli of the donor heart by 24, 27, and 25 seconds, respectively. That the first two responses follow by only 10 and 12 seconds is consistent with the changes which occurred in the rate of perfusion.

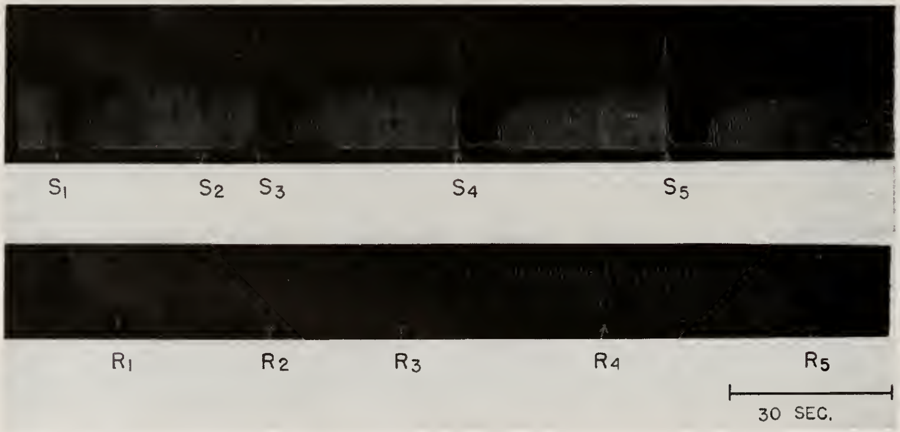

FIGURE 5. Record of series perfusion experiment. Upper tracing is that of the donor heart; lower tracing shows response of receptor heart. See text for further explanation.

D. The effect of tetraethylammonium ions and acetylcholine on the electrocardiogram

Having demonstrated a chemical response to electrotonus, the next logical step was, conversely, to determine the electrical response to treatment with acetylcholine. Ideally this would have meant determining the changes in both resting and action potentials. However, the organization of the fibers in the Venus heart is so irregular that resting potential measurements were impractical. The effects on the ECG were recorded and correlated with simultaneous effects on the amplitude and frequency of the mechanical response. Figure 6 shows simultaneous kymograph and electrograph records taken before treatment (a, a′), after treatment with acetylcholine (b, b′), after partial recovery (c, c′), and after treatment with Et_4N (d, d′). The ECG exposure time is indicated by the signal on the kymograph record. Arvanitaki and Cardot (1933) reported a very similar series of ECGs from the

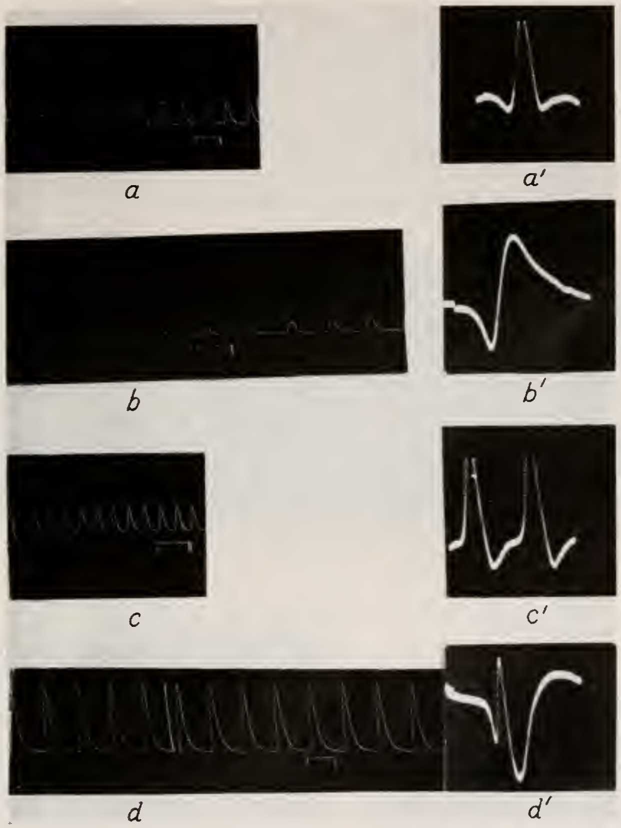

FIGURE 6. Simultaneous mechanical and electrical responses of isolated heart.
a, a′ Normal heart.
b, b′ During application of acetylcholine.
c, c′ After washing.
d, d′ Following the addition of Et_4N.

Helix heart in response to treatment with magnesium ions, and demonstrated that if the waves were analyzed as the sums of two diphasic waves of opposite sign and different speeds of propagation, the complex changes in the observed wave form could be interpreted as changes in the synchrony of the two diphasic components.

The wave form of the ECG follows the type of pattern which Prosser (1950) and Prosser, Bishop, Brown, Jahn, and Wulff (1950) have described as indicating a myogenic heart.

E. Effect of tetraethylammonium ions on electrotonus

The addition of Et_4N to the bath produced significant changes in the response to anelectrotonus. In obtaining the records shown in Figure 7A, the heart was stimulated for 30 seconds at a voltage which would just give 100% inhibition. Following the stimulus, there was a sudden increase in tonus. This was followed by gradual recovery. After the addition of Et_4N, the same voltage failed to produce complete inhibition, as shown by the escape of the beat during the passage of current. The post-anodal responses were augmented by the Et_4N as shown by the 60% increase in tonus immediately following the anelectrotonus, and by the temporary increase in frequency. The overall recovery from anelectrotonus was also faster.

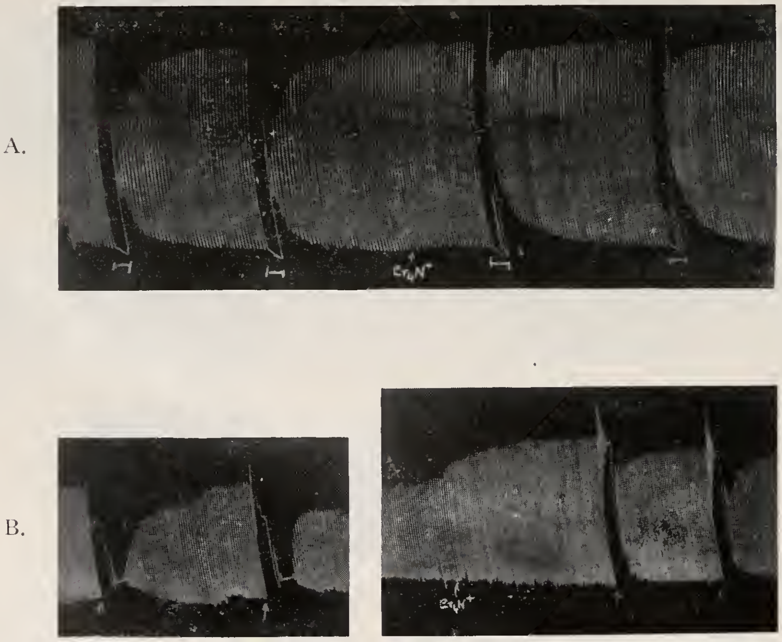

FIGURE 7. A. Record of effect of an anodal current before and after the addition of Et_4N to the bath. B. Record of effect of a cathodal current before and after Et_4N.

Figure 7B shows the effect of Et_4N on catelectrotonus. While two control stimuli showed only a moderate increase in tonus, and a slight tendency to beat during the stimulus, following the application of Et_4N, the increase in tonus was much greater and the beats during stimulation were stronger. The post-cathodal response (*i.e.*, fall in tonus and suppression of beat) was altered so that there was very little change in tonus and less inhibition of the beat.

DISCUSSION

This demonstration of a relationship between the action of specific molecules and that of electrical forces is indirect, and therefore not conclusive. But at the present stage of our knowledge of the factors involved in muscular contraction, and the complex series of events which bring about muscular activity, it is reasonable to postulate a relationship when a striking similarity of site of action and mode of action can be shown. It is in the light of these similarities that the foregoing experiments will be discussed.

Repetitive stimulation of the isolated Venus heart resembles stimulation of the visceral ganglion and direct application of acetylcholine in every respect. The reactions are prolonged by an anticholinesterase, and inhibited by an acetylcholine-blocking agent. The presence of acetylcholine in the heart of *Venus mercenaria* as well as small amounts of cholinesterase (Jullien *et al.*, 1938; Smith and Glick, 1939) makes it reasonable to postulate that the repetitive stimulation of the heart has released acetylcholine. It is not, however, possible to determine from available evidence whether this release is from muscle tissue or from nerve endings. The difference between the response to repetitive stimulation and the responses to DC stimulation indicates that the responses to DC stimulation are not mediated by the release of acetylcholine, but are the result of a direct electrotonic influence on the tissues themselves.

Further demonstration that DC stimulation involves more than the release of acetylcholine from nerve endings is the proportionality of the response with the increase of the duration of the applied current.

That reversal of polarity also reverses the type of response is the most conclusive evidence that this inhibition is not secondary to nerve stimulation. It further suggests that the process which is affected by these currents is itself basically an electrical process, *e.g.*, subject to polarity changes. If increase in tonus and frequency be accepted as criteria of excitation, then catelectrotonus is principally excitatory, and anelectrotonus is inhibitory. Thus, superficially, anelectrotonus resembles the action of acetylcholine and catelectrotonus resembles the action of acetylcholine-blocking agents.

If there be a close relationship between electrical stimulation and treatment with certain ions, then it is reasonable to expect that there will be a chemical response to electrical stimulation as well as an electrical response to treatment with the ion in question. The demonstration of the first condition was realized with the series perfusion experiment. That the response of the receptor heart did not resemble in detail an acetylcholine response does not detract from the thesis. It shows that chemicals are released and that these chemicals are concerned with contraction (and are factors in regulating frequency and amplitude as well as tonus). The demonstration of an electrical response to treatment with acetylcholine was somewhat more direct. A comparison with the analysis of Arvanitaki and Cardot (1933) of the magnesium response of the Helix heart will show that the synchrony of the two diphasic components was affected quite differently by acetylcholine and its blocking agent.

Having demonstrated an interrelation between electrotonus and the acetylcholine system, it now remains to define the closeness of this relationship. The specificity of Et_4N as an acetylcholine—blocking agent in the Venus heart should, by the nature of its effects on electrotonus, help to define the closeness of electrotonus and the acetylcholine mechanism. Tetraethylammonium ions have been shown to antagonize the inhibitory action of anelectrotonus, not only in decreasing its ability to inhibit the beat (Fig. 7A), but, in the case of the post-cathodal response (which is essentially anodal in character), in antagonizing the fall in tonus as well as the suppression of beat (Fig. 7B). Not only is there an antagonism between Et_4N and anelectrotonus, but there is a synergism between Et_4N and catelectrotonus. This is shown in the increase in the cathodal make contraction (Fig. 7B), in the increase in the tendency to beat during the stimulus, and further in the increase in the post-anodal response (*i.e.*, increase in anodal break contraction, and increase in beat frequency seen in Figure 7A). It has already been pointed out that the process affected by electrotonus must be basically an electrical process, since it is reversed by changes in polarity. That Et_4N affects a closely allied process is strongly suggested by the fact that the Et_4N effect is also reversed by changes in polarity. When this evidence is added to the specificity of Et_4N ions in blocking acetylcholine, it seems likely that there is a close functional relation between electrotonus and the acetylcholine system.

The results obtained in the present study, together with earlier findings, lead to the following tentative view of the mechanism of action of acetylcholine in the Venus heart: impulses in cardio-inhibitor nerve fibers ending in the two atrioventricular regions of the heart release acetylcholine from an inactive complex within the smooth muscle fibers themselves. The released acetylcholine plays a role in processes leading to changes in polarity of muscle fiber membranes and doubtless to changes in permeability. Normal contraction and the spread of excitation by way of muscle fibers is interfered with and the amplitude and frequency of beat are reduced, or the heart stops in a relaxed condition, depending upon the amount of acetylcholine set free. Recovery occurs as the acetylcholine is slowly destroyed by the small amount of cholinesterase present and is washed away by the circulating blood.

SUMMARY

1. The relationship between electrical stimulation and inhibition by acetylcholine has been studied in the Venus heart.

2. Stimulation of the heart with direct current produced negative inotropic, chronotropic, and tonotropic effects when anodal, and produced positive chronotropic and tonotropic effects when cathodal. The reversibility of the response with changing polarity is taken as an indication that the step in the regulatory process influenced by electrical stimulation is itself fundamentally electrical.

3. A chemical response to electrical stimulation was demonstrated by the effect on a receptor heart of fluid perfused from a stimulated donor heart.

4. An electrical response to acetylcholine is shown by changes in the ECG. These changes are interpreted as changes in conduction velocity of two propagated monophasic components of opposite sign.

5. The acetylcholine-blocking agent tetraethylammonium bromide is shown to antagonize anodal effects and to augment cathodal effects in this tissue.

6. These interactions between electrical and chemical aspects of the beating of the Venus heart suggest that the acetylcholine system is intimately associated with the electrical aspects of excitation.

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CYTO-EMBRYOLOGICAL STUDIES OF SEA URCHINS. I. THE MEANS OF FIXATION OF THE MUTUAL POSITIONS AMONG THE BLASTOMERES OF SEA URCHIN LARVAE[1]

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Among embryologists in recent years, an attempt has been made to elucidate the cause of morphogenesis on the basis of the activities of individual cells composing the larvae. In a brilliant series of studies begun in 1930, A. R. Moore has endeavored to show, in sea urchin larvae, that blastula- and gastrula-formation can be explained by considering the cohesion of blastomeres by protoplasmic strands on the one hand and colloidal osmotic pressure changes within the blastocoele on the other. Holtfreter's (1943, 1947, 1948) elaborate work on amphibia, in which he attributed prime importance to the "surface coat," follows a somewhat similar plan.

From a recent study of the hyaline plasma layer of sea urchin eggs, however, the authors have arrived at a notion slightly different from Moore's as to the role of this layer, and the present paper presents a critical comparison of the two concepts. In later papers, the results of the repetition of Moore's experiments will be presented, in which practically all Moore's interesting findings are confirmed with some additional facts and the data so obtained reconsidered from the authors' point of view.

In a study of the eggs of the medusa, *Spirocodon saltatrix* (Dan and Dan, 1947), the spinning activity which binds together newly formed blastomeres was investigated. In that form, the entire course of the process could be followed, from the formation of fine processes on the furrow surface to their later amalgamation into a lamella. The process was unaffected by the absence of Ca^{++} ions. These observations raised the question of how the mutual positions of blastomeres are fixed in sea urchin larvae.

In the literature, there are two schools of thought which disagree on this problem. One is the group led by the Andrews (G. F. Andrews, 1897; E. A. Andrews, 1897a, 1897b, 1898) and recently supported by A. R. Moore and his associates (A. R. Moore, 1930, 1945; M. M. Moore, 1932; Whong, 1931). These workers contend that the mutual positions among sea urchin blastomeres are maintained by protoplasmic threads which bind them together. Andrews believed these threads to be the result of a "spinning" process. However, for the present authors, who have followed the entire process of spinning in *Spirocodon,* the bases for their conclusions seem rather unsatisfactory, since none of them succeeded in seeing the threads being spun in the living condition.

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[1] Preliminary notes (in Japanese) covering part of the contents of this paper were published in the Yatsu Jubilee Volume of the Zoological Magazine, vol. 57, pp. 127–131, 1947.

In 1900, Herbst challenged this idea of the Andrews with experimental data. In his well-known experiment using Ca-free sea water, he found that the blastomeres fell apart on the dissolution of the hyaline plasma layer in the absence of Ca^{++} ions. From this, he proposed that the hyaline plasma layer was a sort of "Verbindungsmembran" which binds the blastomeres together. But Herbst did not commit himself to any definite opinion concerning more concrete details of this function.

As a matter of fact, this point continues to remain vague. Chambers (1925) proposes that in *Echinarachnius parma,* the hyaline plasma layer serves simply as a bag, since the blastomeres can be moved freely within it with a microneedle. Gray (1931) maintains a more complicated view. He believes that the hyaline plasma layer of sea urchin eggs is composed of an outer gel part and an inner sol part, the second of which directly surrounds the blastomeres. In another study on the gills of the mussel, *Mytilus edulis,* he successfully demonstrates that the intercellular matrix holds the cells together. Unfortunately, Gray's connection with these two originally separate observations seems somehow to have resulted in a confusion of their component parts, giving rise to the misconception that the inner fluid portion of the hyaline layer of sea urchin larvae acts as a cementing agent between the blastomeres. It is obviously a questionable procedure to assign a cementing role to a fluid substance.

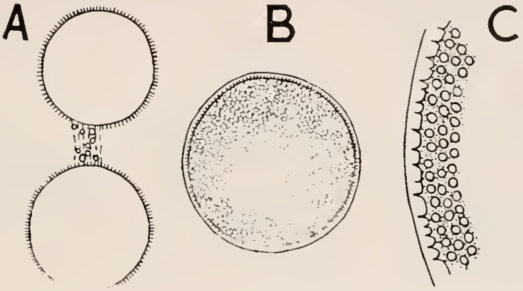

FIGURE 1. (A) A fertilized sea urchin egg in a calcium-free medium showing the halo-layer (after Herbst). (B) A fertilized sea urchin egg in sea water showing radial lines running across the intact hyaline plasma layer (after Gray). (C) Enlargement of (B).

As can be clearly seen in Figure 5, the outer gel part remains extended over the furrow instead of inserting itself between the blastomeres. Therefore, the authors believe it erroneous to think, as Gray does, that the outer gel layer and the inner fluid layer both together make up the hyaline layer. Only the gel part must be considered as the hyaline layer.

Lastly, two things must be mentioned about the structure of the hyaline plasma layer. In his first work on the effect of Ca-free sea water on sea urchin eggs, Herbst (1900) noticed that after the dissolution of the hyaline plasma layer, numerous fine processes remained on the surface of the eggs. He described such a surface with the term "Strahlensaum," which is sometimes translated as "halo-layer." (See Herbst's Plate XVIII and also Motomura's Figure 12, 1941.) Herbst thought that this halo-layer was the hyaline layer itself which had changed its form in the Ca-free medium (Fig. 1 A). On the other hand, Gray (1931) has pointed out that there are many radial lines running across the intact hyaline plasma layer which are really cytoplasmic in nature (Fig. 1 B and C).

The authors propose to demonstrate that Herbst's halo-layer and Gray's radial processes within the intact hyaline plasma layer are identical structures. Moreover, it is easy to imagine that these numerous processes, extending between the inside of the gel layer and the surfaces of the blastomeres, would serve to prevent any mutual shifting of position among the blastomeres. These processes will hereafter be referred to as "attachment fibers."

BASIC FACTS

The opinion expressed above to the effect that the radial striations within the hyaline plasma layer are protoplasmic processes attaching the egg surface to the hyaline plasma layer is a concept of a rather *a priori* nature. The factual evidence in its support is presented below.

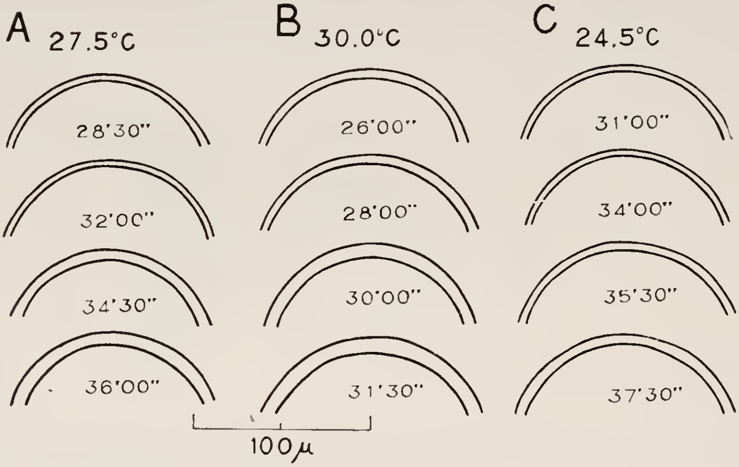

FIGURE 2. Outline drawings of the hyaline layer and the egg contour, by a camera-lucida, of three *Mespilia* eggs showing a sudden increase in the thickness of the hyaline plasma layer immediately preceding cleavage.

1) *Attachment fibers.* Under normal conditions, the hyaline layer becomes clearly visible around the egg about 10 minutes after fertilization. Subsequently, the layer retains a more or less constant thickness. During this time the radial striations are just recognizable within the layer.

A few minutes before the onset of cleavage, however, the layer is suddenly lifted off the egg surface (see Fig. 2) and the striations become more apparent. Figure 4 shows a photograph of the egg of *Mespilia globulus* in this condition. On examination, it is revealed that this apparent lifting off of the layer is really due to an accumulation of fluid between the layer and the egg surface. As a result of the accumulation of this fluid, the hyaloplasmic envelope is inflated, which, in turn, stretches the attachment fibers. This stretched condition of the fibers makes the striations stand out particularly clearly (Fig. 3 B).

2) *Protoplasmic nature of the attachment fibers.* Staining the eggs with neutral red and observing them through a green filter [2] greatly improves the optical differentiation of the attachment fibers. Figure 5 illustrates the two-cell stage of a *Mespilia* egg so treated. The material shown in Figure 5 is in the late two-cell stage, when the blastomeres are rounding up in preparation for the second cleavage. It is obvious that the rounding blastomeres are stretching the attachment fibers in the vicinity of the furrow.

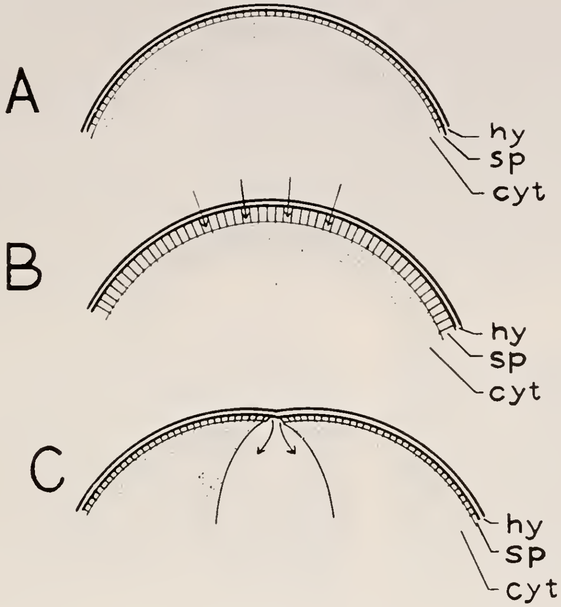

FIGURE 3. Explanatory figures for illustrating the movement of the intra-hyaloplasmic fluid. (A) Condition of the hyaline layer during the monaster and the streak stages. The amount of the fluid is small or practically none. (B) Within three minutes prior to cleavage, fluid which comes from outside through the hyaline layer accumulates between the layer and the cell surface. The hyaloplasmic envelope is thus inflated and consequently the attachment fibers are stretched. (C) After cleavage, the fluid comes around into the space between the freely movable apposed surfaces, releasing the tension on the attachment fibers. Fibers on both sides of the cleavage furrow slant toward the cleavage plane, showing that their bases are being pulled apart. Hy: hyaline layer; sp: fluid space; cyt: cell body.

Over-staining with neutral red often causes a roughening of the fibers, as is illustrated in Figure 6. This condition is convenient for showing the protoplasmic nature of the fibers, because in the thickened state they are not only more easily traced to the egg surface but they even acquire the same coloration as the cytoplasm. On these bases, it seems justifiable to identify the radial striations of the hyaline layer with the halo in a Ca-free medium.

3) *Adherence of the blastomere surface to the hyaline plasma layer by means of the attachment fibers.* As was stated in the previous section, fluid accumulates between the egg surface and the hyaline plasma layer just before the first division.

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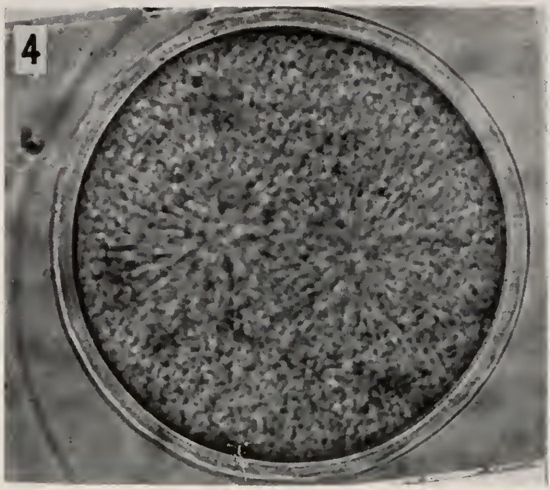
[2] Filter: Riken's "Luminas filter" No. 110. Plate: Fuji panchromatic process.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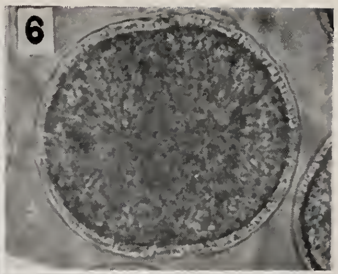
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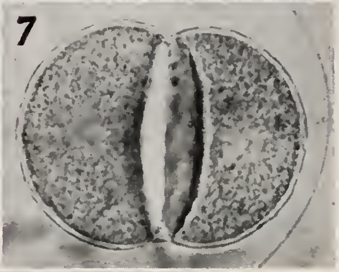
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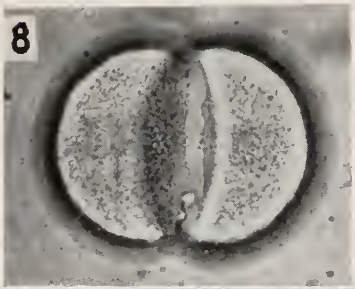
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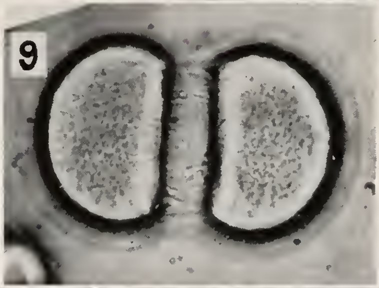
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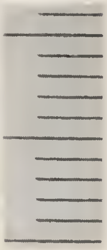

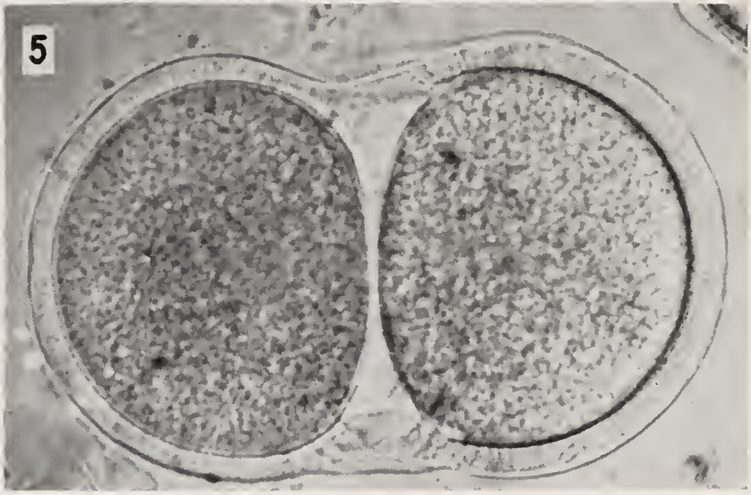
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FIGURE 4. Egg of *Mespilia globulus* immediately prior to the first cleavage. The apparent thickness of the hyaline plasma layer has increased, owing to the accumulated fluid under the

Although the cause of this accumulation will be dealt with in a later paper, the reader is asked to accept, for the time being, the idea that it is due to the development of a colloid osmotic pressure within this space.

During the one-cell stage, the accumulated fluid is automatically distributed evenly around the egg cell. However, when the mid-interkinetic stage of the two-cell stage is reached, its distribution changes fundamentally. At this stage, contrary to the contention of Andrews and Moore, no spinning threads whatsoever can be found between apposed surfaces of the two blastomeres. As a result, the apposed surfaces are not bound together but are quite free to separate. They are therefore pulled apart by the tension of the attachment fibers (Fig. 3 A, B, C), while the fluid which had been surrounding the egg cell streams into the space between the blastomeres.

Another factor which should be borne in mind is that the hyaloplasmic envelope at this stage is inflated in comparison with the non-cleaved stage as the result of the fluid accumulation. Consequently when the outer surface of the blastomeres is pulled toward the hyaline plasma layer, it must be expanded to equal the new size of the hyaline plasma layer. The persistence of the connection between the blastomere surface and the hyaline plasma layer produces the result shown in the surface view of the furrow region during the interkinesis (Fig. 8), where the "rims" of the cup-shaped blastomeres, obviously under some tension, are stretched out into thin films of cytoplasm.

At the end of the interkinetic stage, as the development of the diasters draws the cytoplasm toward the centers of the blastomeres, the granular endoplasm is completely withdrawn from the thin apposed rims (Fig. 9), leaving only the transparent surface layer still attached to the inside of the hyaline plasma layer. Evidence that the blastomere surface retains its attachment is found at each succeeding interkinesis, when the disappearance of the mitotic apparatus and consequent diminution in turgidity of the blastomeres permits granular cytoplasm to flow back into the thin peripheral areas, as can be seen in the surface view of the second interkinesis (Fig. 11).

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layer. The protoplasmic processes which appear as radial striations are being stretched between the hyaloplasmic gel layer and the egg surface.

FIGURE 5. Later two-cell stage of *Mespilia* when the blastomeres are rounding up for preparation for the second cleavage. The material is stained in neutral red and observed through a green filter. Notice that the protoplasmic processes are more evident. Those around the cleavage furrow are pulled out by the rounding cells.

FIGURE 6. Egg over-stained by neutral red in which the protoplasmic processes have become thicker and coarser.

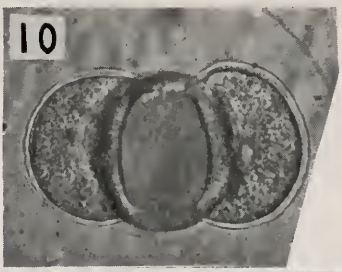
FIGURE 7. Optical section of the two-cell stage of *Mespilia*, showing a lens-shaped space between the two blastomeres. The white patch on the left blastomere is a reflection of light from the surface. In this stage, the apparent thickness of the hyaline plasma layer decreases again.

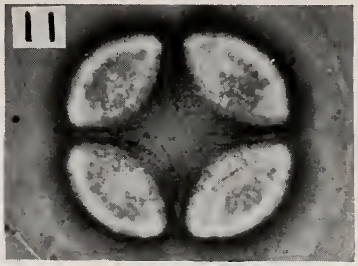
FIGURE 8. Surface view of the egg shown in Figure 7, showing the thin tongue of cytoplasm along the contact line between the two blastomeres.

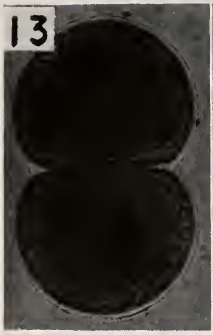
FIGURE 9. Surface view of the two-cell stage in which the two apposed surfaces separate widely. Note that the cytoplasm of the blastomeres is still adhering to the hyaline plasma layer.

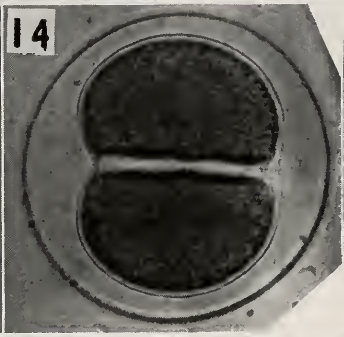
Scale shown on the left is common for Figures 6–9, the smallest division being 10 micra.

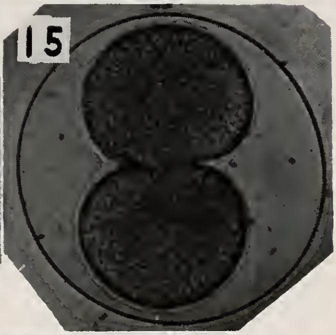
During the interkinetic stage following the second cleavage the fluid space in the center between the four blastomeres becomes larger. In *Mespilia,* because of the

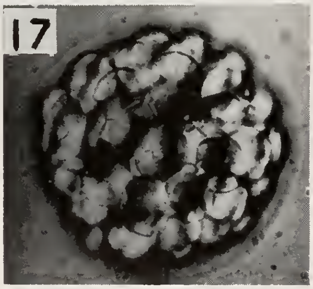












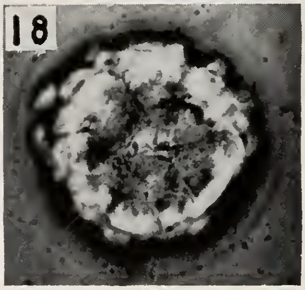


FIGURE 10. Optical cross section of a four-cell stage of *Mespilia globulus,* showing a large fluid space at the center of the four blastomeres.

FIGURE 11. Surface view of the junction of four blastomeres of *Mespilia.*

FIGURE 13. The interkinetic stage of the two-cell stage of *Strongylocentrotus pulcherrimus.* The fertilization membrane has been removed. The two blastomeres are in close contact without any such between-space as is found in *Mespilia.*

FIGURE 14. The two-cell stage of *Strongylocentrotus* treated with a hypertonic medium, showing the appearance of a space between the two cells. A glassy spindle remnant is in the center of the space.

FIGURE 15. A two-cell stage of *Strongylocentrotus* previously treated with isotonic urea, in a hypertonic medium. The softened hyaline layer is pulled in with the shrinking blastomeres without forming the lens-shaped space.

FIGURE 17. Surface view of a morula of *Clypeaster japonicus* during a division cycle. Note that the cells bulge out, the intercellular space appears and the whole larva becomes bumpy on the surface.

FIGURE 18. The larva shown in Figure 17 during the following interkinetic stage. The slackened cells are spreading along the hyaline layer and the intercellular space is abolished.

transparency of the blastomeres, this space can be photographed through the cell bodies. Figure 10, shows such a large space and it also shows how the "rims" of cytoplasm are encircling the fluid space. The surface view at the junction of the four blastomeres is shown in Figure 11.

4) *Inflation of the hyaloplasmic envelope and a consequent dispersal of the blastomeres.* If 4- to 16-celled embryos of *Mespilia* from which the fertilization membranes have been removed are put into 50% or 70% sea water, the hyaline plasma layer becomes inflated to a great extent and the blastomeres are carried with it away from each other. Figure 12 shows that, in spite of the volume increase of the blastomeres in the hypotonic medium, the cells are widely separated from one another. The dilution in the salt concentration obviously causes a softening of the hyaloplasmic gel. Such a decrease in the tensil strength of the layer would result in an increase in equilibrium volume even when the contained colloid remains constant in amount. The softening of the gel is further attested to by the fact that the hyaloplasmic layer under these conditions deviates from a spherical shape, owing to the pull by the blastomeres.

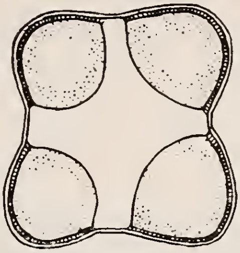


FIGURE 12. Four-cell stage of *Mespilia globulus* put in 50% sea water. The hyaloplasmic envelope is softened and expanded. The blastomeres are carried away from each other as the envelope enlarges.

5) *Experimental induction of a lens-shaped space between the blastomeres.* In the eggs of *Strongylocentrotus pulcherrimus,* the amount of the fluid which accumulates between the blastomeres during the interkinetic stage of the two-cell stage is not so large as in the case of *Mespilia globulus.* As a result, the two blastomeres are nearly always touching at the cleavage plane (Fig. 13). If the adherence between the hyaline plasma layer and the blastomere surface also exists for *Strongylocentrotus,* there ought to be a way to induce a lens-shaped space also in this species by artificial means. As was pointed out above, the appearance of the lens-shaped space in *Mespilia* is caused by the inflation of the hyaloplasmic envelope. However, the same end will be achieved by decreasing the volume of the blastomeres while keeping the hyaloplasmic envelope unchanged. Such a condition should be obtainable by putting the two-cell stage of *Strongylocentrotus* into a hypertonic medium, because in the hypertonic medium, the blastomeres would shrink while the volume included within the hyaloplasmic envelope would not be affected, since the layer is freely permeable to salts. This expectation is borne out, as is shown in Figure 14.

6) *Rigidity of the hyaline layer and the appearance of the lens-shaped space.* It can further be shown that the rigidity of the hyaline plasma layer is an essential

factor for the appearance of the lens-shaped space in *Strongylocentrotus.* If fertilized eggs with the fertilization membranes intact are put into an isotonic urea solution, the hyaline layer swells and fills the perivitelline space. On being returned to sea water, however, the hyaloplasmic substance shrinks again and the layer is re-formed, although it is slightly roughened and softer than normal. When the eggs with softened hyaline layer are exposed to a hypertonic medium (15 gr. of NaCl added to 1 liter of sea water), the hyaloplasmic layer is pulled in with the blastomere surface and no lens-shaped space appears between the blastomeres. This condition is shown in Figure 15.

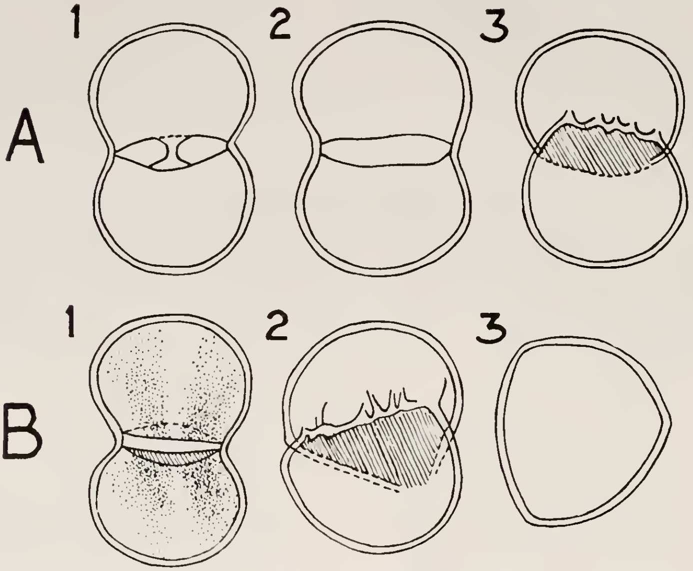


FIGURE 16. Two-cell stage of *Strongylocentrotus pulcherrimus* treated with a hypertonic medium at early (A) and later (B) interkinetic stage. A-series: 1) Appearance of a lens-shaped space with a spindle remnant. 2) A little later. The spindle remnant has dissolved. 3) Oblique view. The apposed surface is roughly circular in shape. B-series: 1) Surface view. The blastomeres are triangular in shape. 2) Oblique view. The apposed surfaces are also triangular. 3) Polar view of one of the blastomeres. This is also triangular, indicating that the blastomeres are tetrahedral in shape.

7) *Change in the tendency for forming the lens-shaped space during interkinesis.* When the time of exposure of *Strongylocentrotus* eggs to the hypertonic medium is shifted during the interkinesis of the two-cell stage, significantly different results are obtained. If the hypertonic treatment is given immediately after cleavage, a typical lens-shaped space is formed as has been described (see Fig. 14). But if the transference to hypertonic sea water is made from 15 to 20 minutes later, although a gap appears between the two blastomeres to some extent, a more conspicuous effect is that the entire blastomere takes a roughly tetrahedral shape (Fig. 16).

This marked difference in the forms assumed by the blastomeres under the same treatment must be attributed to the change which takes place in the consistency of the apposed surfaces during the interkinetic period. There are reasons to think that the apposed surfaces are weak immediately after the completion of a cleavage, gradually, however, attaining the same consistency as that of the rest of the cell surface as the time passes.[3] As the result of this change, the inclination for the apposed surfaces to be selectively indented disappears, and the blastomeres tend to shrink uniformly over their entire surfaces. An important point is that when this happens, the shrinkage force is directly transmitted to the hyaline plasma layer by the mediation of the attachment fibers. But the hyaline plasma layer is inelastic and its surface area is fixed. The inevitable result is that the larva assumes a tetrahedral shape, since among objects with the same surface area, a tetrahedron includes the minimal volume. From the above observations, it can be said that if allowance is made for the change in strength of the apposed surfaces, the notion of the connection between the elastic cell surface and the inelastic hyaline plasma layer can admirably explain the rather complicated behavior of the blastomeres in a hypertonic solution.

Discussion

Although the work reported in this paper was originally planned as a part of our serial study on the behavior of the cell surface during cleavage, in developing, it led our interest into a new channel. In this sense, the present paper is a connecting link between the previous series on cell division and a new one on cyto-embryology. As a result, some phases connected with the former series must also be discussed.

(1) *Considerations on surface behavior during cleavage*

The new notion of the adherence of the blastomere surface to the hyaline plasma layer necessitates re-consideration of the results of the kaolin experiments previously reported. In the first paper of the kaolin experiment (Dan, Yanagita and Sugiyama, 1937), the behavior of the hyaline plasma layer and that of the naked cell surface during cleavage were reported in the eggs of *Mespilia globulus*. The results indicated that while the various regions of the denuded cell surface behave in a manner characteristic to their topographical positions in reference to the spindle, the hyaline plasma layer shows a slight and uniform change over the entire surface with some exceptions for the furrow region. This uniform change in the hyaline layer was interpreted in terms of Gray's dual structure of the layer and it was suggested that the hyaloplasmic gel must be slipping over the underlying cell surface, although the cell surface itself would still be moving in a regionally characteristic fashion. As a result of the present investigation, this interpretation has been shown to be erroneous.

It is, however, now possible to imagine the behavior of the cell surface under an intact hyaline layer in the following manner. At division, the movement of the cell surface would ordinarily be prevented because of its connection to the hyaline layer, except when the change on the naked surface becomes strong enough to overcome the rigidity of the hyaloplasmic gel. This latter situation arises only at the cleavage plane. When the furrow region of the protoplasmic surface shrinks during the initial stage of division, the overlying hyaline layer is made to wrinkle. In later stages of division when the protoplasmic surface cuts in as a furrow, the overlying hyaline layer is often stretched and pulled in with it as long as the attachment fibers do not break. In short, because of this fixation of the surface, when a furrow cuts in, the material forming the newly formed furrow surfaces must consist of the stretched-out material derived from a very narrow strip which girdled the equator of the spherical stage. This point is important for an analysis of the discrepancy in opinion between Motomura and Dan on the division mechanism which will be given in a future paper.

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[3] This problem will be taken up separately in another paper.

(2) *Considerations on the mechanism of cleavage*

Observing the cleavage of *Arbacia* eggs in a hypotonic medium (65% sea water), Scott (1946) noticed an unusual elongation of the stalk which is left between the separating blastomeres. Scott, who believes that this stalk is composed entirely of gel, interprets this phenomenon as an indication of an extraordinary power which the equatorial surface possesses of contracting by gelation. On this basis, he throws doubt on the author's hypothesis, saying (p. 284), "Dan's hypothesis is not in accord with the observations presented here concerning the continued elongation of the cleavage stalk in hypotonic sea water for it is impossible to see how the astral suction mechanism could explain the further constriction of a long, completely gelated stalk."

As a matter of fact, the observation is a very good example of astral cleavage. According to the author's theory, cell division is achieved through separation of two asters by an autonomously elongating spindle. It was already mentioned that in this elongation there is always found a slight over-shoot in order to secure a clear-cut separation of the blastomeres (Dan, 1943, p. 318). By over-shoot, it is meant that the elongating spindle becomes longer than just sufficient to separate the blastomeres so that the spindle is exposed at the middle, forming the stalk. Under certain experimental conditions and with special materials, this stalk is observable.

As soon as cleavage is complete, however, the stalk begins to dissolve, passing through a beaded stage and eventually separating into many droplets. Scott traced the fate of the stalk only to this beaded stage. Thinking that the beaded condition is an expression of exaggerated contraction, he concludes (p. 285), "the observations made in this paper on the *continued* [4] constriction of small stalks after they consist entirely of gelated material are taken as strongly favoring the constricting ring theory of Lewis." On the contrary, the authors believe that the beaded condition is actually an indication of liquefaction.

The over-shooting and later liquefaction of the spindle are phenomena of constant occurrence as long as a cell divides, and are not such haphazard things easily affected by environmental changes as Scott suggests. But unless a special material such as *Mespilia* which forms an extraordinarily wide lens-shaped space is used, the stalk is usually hidden from sight as in *Strongylocentrotus* shown in Figure 13. It comes into view only when the external resistance offered by the hyaline layer is removed either by its dissolution or softening. Scott used variously a Ca-free medium, hypotonic medium and low temperature to obtain such an effect.

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[4] Our italics.

Certainly either of the first two of his conditions would result in removing or softening the hyaloplasmic gel.

(3) *Considerations on the cleavage pattern*

(a) The formation of the lens-shaped space in sea urchin eggs reminds one of the same phenomenon occurring in other forms. This is often reported in Molluscs: in the eggs of the snail, *Lymnaea*, and so far as the authors are aware, most strikingly of all in *Dressensia polymorpha*, studied by Meisenheimer (1900).

It must be mentioned here that in the eggs of the medusan, *Spirocodon saltatrix*, which have absolutely no extraneous coats and in which a typical spinning activity takes place between the blastomeres, a lens-shaped space appears when the eggs are put into a hypertonic medium during the interkinetic stages. An explanation of this phenomenon, even in the absence of an enveloping layer, is conceivable if the apposed surfaces are found to be sufficiently weaker than the other surface areas. Further investigation of this point is needed.

(b) Concerning the surface area of blastomeres, Errera (1886) and Thomson (1887) were the two earliest investigators to point out the conformity of the cleavage patterns of animal eggs to the Law of Minimal Surface, and Robert (1902) imitated cleavage patterns of the spiral type with soap bubbles. Although the Errera-Thomson Law involves a very keen analysis, it must be admitted that the Law is rather an admirable description of the cleavage pattern of the spiral type than an explanation of the phenomenon. But what the Law does bring out clearly is the fact that the cleavage pattern of sea urchins as it is manifested in the four-cell stage decidedly does not conform to the principle of the minimum surface (see Fig. 10). The force by which sea-urchin blastomeres resist the Law is supplied by the adherence of their surfaces to the common envelope (the hyaline plasma layer), which is an inelastic gel.

(4) *Considerations on the spinning theory*

G. F. Andrews was the first investigator to report on the spinning activities in starfish and sea urchin eggs (1897). She pictured numerous "spin threads" coming out from the furrow sides at the first cleavage as well as at the following ones. She also described the irregular contours of the cells around the "cleavage cavity pore" at the time of its closure and interpreted this as a result of spinning among the neighboring cells.

E. A. Andrews expanded the observations to other forms and reported his results in three papers (1897a, 1897b, 1898). Leaving aside a discussion of fixed materials for the present, what he found in living eggs of both *Serpula* and *Yoldia* was not the anastomosis of protoplasmic strands but only radially arranged processes reaching the enveloping "cuticle." In other words, the condition he observed coincides perfectly with what the authors find in sea urchin eggs.

More recently, Moore (1930) supported the spinning hypothesis with other lines of evidence. He believes that there are two kinds of material connections between blastomeres: the primary and the secondard bridges. His single primary bridge is formed at the point where the blastomeres were last connected. The numerous secondary bridges between daughter cells are characterized by their appearance *only* when the blastomeres are brought into contact with each other.

Concerning the primary bridge, judging from Moore's descriptions and also from the references cited, there is no room for doubt that he is referring to the remnant of the division spindle. But the present authors cannot agree with his opinion that the primary bridge remains through many division cycles. In a Ca-free medium, it is easy to see the disintegration of the spindle remnant during the interkinetic stage. In normal sea water, in which the blastomeres come into close contact, the spindle remnant can be seen by the hypertonic treatment reported above. If the eggs are treated immediately after cleavage, a highly refractive glassy spindle is visible, as shown in Figure 14, while if treated later, no such structure can be seen.

Concerning the secondary bridges, the most important point is the fact that Moore himself rejects the possibility of the spinning activity in Andrews' sense. Moore writes (p. 196), "It should be specifically stated that in our experience functional cell-bridges arise only as the result of contact between the blastomeres. They are never spun across an open space as stated by E. A. and G. F. Andrews." Considering the fact that the Andrews' finding has never been confirmed, it is almost certain that the possibility of the spinning activity in their sense is ruled out, at least for early segmentation stages of sea urchins.

Now, Moore insists on the point that the secondary bridges are formed only when the blastomeres are brought in contact. His student Whong (1931) showed that such secondary bridges can also be made to appear between two eggs. However, as long as contact is said to be indispensable for the establishment of the secondary bridges, it is impossible to see the bridges in their formation. Consequently, the only way left is to make a careful observation of the formed bridges and judge whether they are really living cell constituents or the passive products of adhesion. Unfortunately, Moore's paper includes hardly any description of them except that they are hyaline. Whong sketched them as numerous fine threads. M. M. Moore mentions (1932, p. 492) that in sea water they are broad and granular; in Ca-free medium, narrow and colorless. This much description is insufficient to clinch their argument because a "broad granular" projection in sea water may very likely correspond to the rims of cytoplasm which the present authors showed in Figures 8 and 11, and a "narrow and colorless" projection can easily be an extraneous structure resulting from adhesion. But before proceeding further, we have to consider their third piece of evidence.

According to A. R. Moore, if a larva which is devoid of the hyaline plasma layer is allowed to develop to a stage corresponding to a blastula, the blastomeres form a flat plate or sheet. Furthermore, the intercellular spaces expand at the division of the cells while they vanish during interkinesis, and the whole plate alternately curls up and flattens, exhibiting such movement as might be called systole and diastole, with each division cycle. He attributes this change to the contractility of the cell-bridges.

Obviously, the optimism of Moore's group arises from their belief that their materials are really free from any trace of hyaloplasmic substance. But simply for the sake of argument, if the existence of a hyaloplasmic residue around the eggs is imagined, Moore's findings can be strung into a different but equally complete story. In the first place, Moore believes that the primary bridge remains through many division cycles, an opinion with which the authors have already expressed disagreement. But when an egg coated by a trace of the hyaloplasmic substance cleaves, the coating layer often accumulates around the connecting stalk and remains for a long time even after the spindle remnant dissolves. Herbst's (1900) figures (Plate XVIII, Figs. 1a–1i) illustrate such an accumulation.

Secondly, if the existence of such a gelatinous coating is imagined, the appearance of the secondary bridges on contact would be a most natural result. It is regrettable that Moore and Whong did not attempt to induce the formation of secondary bridges by application of some inanimate surface such as a glass rod to the egg surface.

Finally, the plate formation in later stages suggests most forcibly the probable existence of such a gelatinous covering, for when a larva is thoroughly washed in a Ca-free medium, the blastomeres simply pile up, never forming a sheet (Motomura, 1941, Figs. 9, 10, 11). Herbst (1900) writes that if a larva reared in normal sea water is transferred to a Ca-free medium, the larva first turns into a plate and then the cells separate. A phenomenon corresponding to the "systole and diastole" of Moore can actually be seen in a morula developing in sea water, which the authors interpret as a direct result of the presence of the hyaline plasma layer and the attachment to it of blastomere surfaces. Two photomicrographs of a morula of *Clypeaster japonicus* are included here (Figs. 17 and 18). When division occurs, the blastomeres round up and the bulge of each cell is apparent in the outline of the hyaline plasma layer, while the intercellular spaces become more apparent. Diastole corresponds to the interkinetic stage in which the blastomeres are slackened. The slackened cells are now pulled back by the hyaline plasma layer by the attachment and spread along the inner surface of the layer. The inter-cellular spaces are filled in.

In short, as far as Moore's data are concerned, the chance seems to be even in favor of both their and our contentions. Therefore, in order to decide how much guarantee they have that the material is free from a coating of any sort, the last resort left is to examine their method of experiment. Fortunately, M. M. Moore's paper gives a full account of the method adopted. She says that if the eggs of *Paracentrotus lividus* and *Echinus microtuberculatus* are washed in the unfertilized condition with urea or glycerine and are later fertilized in sea water, they give rise to larvae without the fertilization membrane. If they are treated with these agents during the formation of the fertilization membrane, they are quickly killed. If the treatment is deferred too long, the fertilization membrane will not dissolve. "About 10 minutes after normal fertilization membranes have appeared, the eggs are washed in 10 cc. of 1.2 molecular solution of glycerine or urea of pH 8.0, then transferred to another watch-glass containing the same solution. In from 2 to 5 minutes, depending upon the female furnishing the eggs, the jelly and the fertilization membranes swell and dissolve, allowing the eggs to fall out onto the bottom of the dish. *When about half the eggs are free,*[5] they are washed in sea water, then transferred to another dish of sea water where they develop" (p. 489–490).

As is clear from the above quotation, they have only the minimal guarantee since they transferred the eggs from the nonelectrolyte solution to sea water when the dissolution of the substance is barely beginning. The authors do not hesitate to admit from their own experience that non-electrolyte solutions are far more potent than a Ca-free salt solution for suppressing the formation of the hyaline plasma layer. But they are afraid that Moore and his associates were too optimistic in trusting the power of the non-electrolyte solutions.

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[5] Our italics.

It must be mentioned, however, that Moore's group is provided with an objective method to test whether or not the hyaloplasmic residue persists. According to M. M. Moore, the residue, if it remains, can be made visible by adding a small amount of 2.5 molar NaCl solution to the medium (2 or 3 drops in 10 cc.). By means of this technique, she found the residue around Ca-free washed eggs, while on the urea-treated eggs, nothing was seen. Unfortunately, in spite of the ingenuity of this procedure, their results are not guarded against the possibility of a further secretion of the hyaloplasmic substance as the development continues in sea water.

The final point to be mentioned is that one of the reasons for Moore's great insistence on the protoplasmic nature of the spinning processes is that he considers that only the existence of such protoplasmic unity among the blastomeres would allow them to be organized into a harmonic larva. In order to refute his theory, then, we have to show that our concept can also explain the harmonious development of a larva. Such an attempt will be found in the following paper.

The authors wish to express their indebtedness to the staff of the Misaki Marine Biological Station for use of the Station's research facilities. The present work was supported by the Research Expenditure of the Ministry of Education.

SUMMARY

1. The proposal made by the Andrews and A. R. Moore that sea urchin blastomeres are bound together by means of protoplasmic strands is rejected and the notion is advocated that sea urchin blastomeres are stuck to the inside surface of the hyaline plasma layer by attachment fibers radiating out from the blastomere surface. This mode of fixation secures definite relative positions among the blastomeres. Evidences are put forward indicating the above relation.

2. Possible sources of errors in the spinning theory of Andrews and Moore are analyzed.

3. Points concerning the movement of the cell surface during cleavage and the pattern of cleavage are considered.

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CYTO-EMBRYOLOGICAL STUDIES OF SEA URCHINS. II. BLASTULA STAGE[1]

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In the foregoing paper of this series (Dan and Ono, 1952), evidences were put forward showing the connection between the hyaline plasma layer and the cell surface by means of "attachment fibers" (protoplasmic processes radiating out from the cell surface). In that paper, it was also pointed out that a fluid accumulates between the hyaline layer and the cell surface preceding each cleavage. The accumulated fluid, in turn, inflates the hyaloplasmic envelope and thus stretches the attachment fibers. Since this phenomenon has an important bearing on the content of the present paper, a more detailed account will be given.

THE ACCUMULATION AND MOVEMENT OF THE FLUID

Figures 1–11 illustrate the change in the apparent thickness of the hyaline plasma layer during the first and the second cleavages in *Strongylocentrotus pulcherrimus.* These photomicrographs were taken of the same embryo at successive times. The fertilization membrane was removed by shaking immediately after its formation. The first and second photographs were taken three minutes apart. In the second, flattening of the equatorial surface for the furrow formation is just beginning (on the right side). During the three minute interval, the intra-hyaloplasmic space suddenly increases. This is due to the inflation of the hyaloplasmic envelope as the result of the fluid accumulation.

In Figures 3 and 4, the elongation of the cell body and the formation of shallow cleavage furrows are taking place. During this time, the interspace between the egg surface and the hyaline layer becomes narrower at the polar region but wider at the equatorial region. At the latter region, the hyaline layer even wrinkles. Such changes in the width of the interspace are actually due to a shifting of the fluid from one locality to another within the hyaloplasmic envelope.

Such a flowing of the fluid, and the wrinkling of the hyaline layer, are easily understandable from the division mechanism of sea urchin eggs previously proposed by the author (K. Dan, 1943; J. C. Dan, 1948). When the asters begin to travel away from each other, the cell body elongates in the direction of the spindle. This elongation squeezes out the fluid at the polar region, resulting in a narrowing of the interspace. Around the furrow region, on the other hand, the space widens, since the underlying cell surface sinks in, forming the furrow and pulling the hyaline layer with it. Moreover, during the initial stage of furrow formation, wrinkles

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[1] The present research was supported by the Research Expenditure of the Ministry of Education, for which the author's thanks are due.

often appear in the hyaline layer as a result of the shrinkage of the cell surface, to which the layer is bound by means of the attachment fibers.

In Figures 5 and 6, this tendency is more exaggerated. A point to be noticed is the fact that the interspace at the polar region practically disappears and the real thickness of the gelified hyaline layer becomes apparent, coinciding with that observed before the accumulation of the fluid (see Fig. 1). In Figure 7, all the fluid is squeezed out of the polar surface and comes to the equatorial region between the two blastomeres. At this stage, the gel layer at the poles seems to be even thinner than in Figure 1. This is because the layer is now covering a larger area than before, partly because of the accumulation of the fluid, but mostly because of the deviation from the spherical form. As a matter of fact, during the interkinetic stage which is shown in Figure 8, when the two blastomeres come closer together, the hyaline layer more or less regains the thickness as in Figure 1. Under this condition, although the two blastomeres look as if they are in close contact, an appreciable quantity of the fluid must still be distributed between the apposed surfaces.

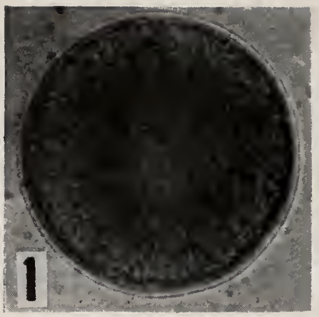
Now in Figure 9, which was taken shortly before the beginning of the second cleavage, it is obvious that the intra-hyaloplasmic space has, for the second time, increased all over the surface. This clearly indicates that a new addition of the fluid takes place once again in connection with cleavage. When the embryos go into the four-cell stage, practically the total thickness of the hyaline layer on the periphery can be attributed to the gelified layer, while a conspicuous space appears at the center (see Fig. 10). During the interkinetic period of the four-cell stage (Fig. 11) the fluid is also seen between adjacent cells.

In short, as the number of the blastomeres increases, the fluid progressively collects in the center. A point of utmost importance is that if such a fluid accumulation is kept up at each cleavage cycle, the hyaloplasmic envelope will become larger and larger and the blastomeres which are attached to the hyaline layer will be carried outward with the expanding envelope, leaving a large fluid space in the center. Such an arrangement of the blastomeres would eventually produce the form of a blastula.

Gray (1931) is fully aware of the movement of the intra-hyaloplasmic fluid. In his interpretation, however, this fluid is considered to be an essential element of the hyaloplasm, while the author thinks it is entirely extraneous to the living system.

The Mechanism of Fluid Accumulation

How the fluid accumulates under the hyaline plasma layer is the next question. At present, nothing definite can be said in a positive sense, although it is possible to prevent the accumulation by an artificial means. This is to add gum arabic to the surrounding sea water. It is obvious that the essential feature of the addition of gum arabic must be the development of a colloid osmotic pressure. Therefore, it is justifiable to think that the accumulation of the fluid under natural conditions must also be caused by the development of a colloid osmotic pressure within the hyaloplasmic envelope. In other words, the same mechanism as that which operates in the elevation of the fertilization membrane is involved in the accumulation of the fluid (Loeb, 1908). Just how such colloid is supplied—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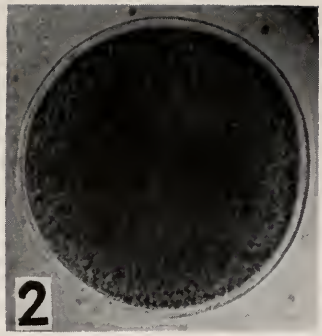
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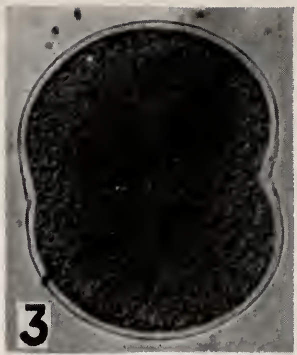
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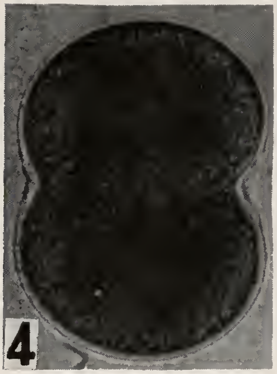
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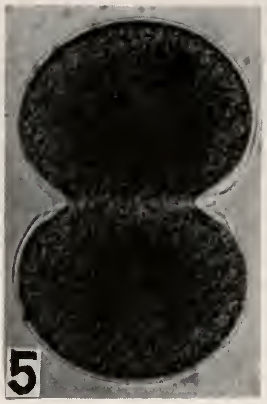
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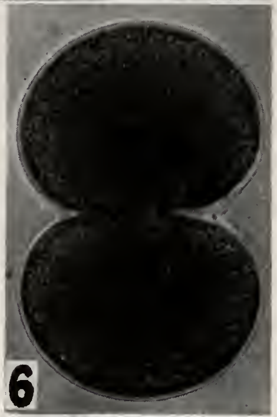
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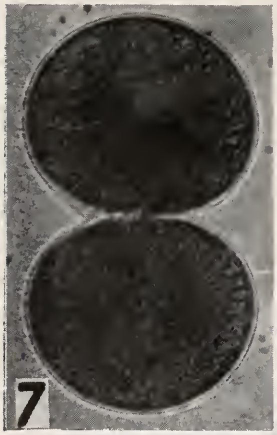
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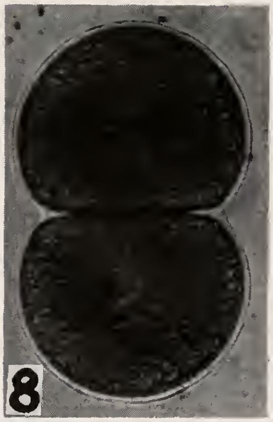
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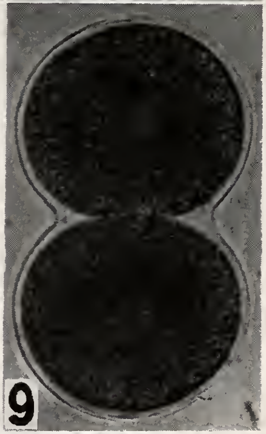
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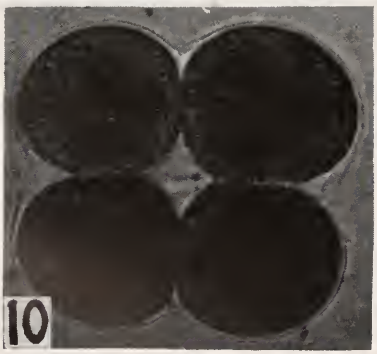
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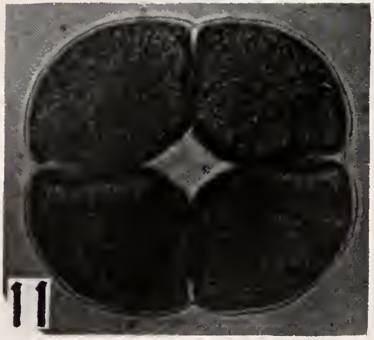
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whether it is secreted by the cell or liberated by the peptisation of the surface substance—cannot be said with certainty at present.

BLASTOCOELIC FLUID

From what has been said in the first section, it can be inferred that the fluid which accumulates around the non-cleaved egg becomes the blastocoelic fluid in later stages. If this statement be true, an environmental condition which modifies the amount of the accumulating fluid in the one-cell stage will, at the same time, cause a similar modification in the amount of the blastocoelic fluid. With the aim of testing this proposition, four cultures of *Mespilia* eggs were made containing, respectively, 4%, 2%, 1% and 0% of gum arabic in sea water.

TABLE I

	Concentrations of gum arabic in sea water							
	0%		1%		2%		4%	
Culture	Volume of hyaline fluid	Volume of blastocoelic fluid	Volume of hyaline fluid	Volume of blastocoelic fluid	Volume of hyaline fluid	Volume of blastocoelic fluid	Volume of hyaline fluid	Volume of blastocoelic fluid
No. 1—10 indiv.	$64.0 \times 10^3\ \mu^3$	$1518.7 \times 10^3\ \mu^3$	$60.1 \times 10^3\ \mu^3$	$809.1 \times 10^3\ \mu^3$	$42.6 \times 10^3\ \mu^3$	$328.5 \times 10^3\ \mu^3$	$5.8 \times 10^3\ \mu^3$	$228.1 \times 10^3\ \mu^3$
No. 2—10 indiv.	$157.6 \times 10^3\ \mu^3$	$1912.9 \times 10^3\ \mu^3$	$100.7 \times 10^3\ \mu^3$	$834.6 \times 10^3\ \mu^3$	$63.4 \times 10^3\ \mu^3$	$470.6 \times 10^3\ \mu^3$	——	——
No. 3—10 indiv.	$231.4 \times 10^3\ \mu^3$	$3188.8 \times 10^3\ \mu^3$	$138.3 \times 10^3\ \mu^3$	$993.8 \times 10^3\ \mu^3$	$87.9 \times 10^3\ \mu^3$	$699.0 \times 10^3\ \mu^3$	$77.2 \times 10^3\ \mu^3$	$325.1 \times 10^3\ \mu^3$
No. 4—10 indiv.	$153.5 \times 10^3\ \mu^3$	$2674.4 \times 10^3\ \mu^3$	$93.8 \times 10^3\ \mu^3$	$1140.1 \times 10^3\ \mu^3$	$77.8 \times 10^3\ \mu^3$	$1010.8 \times 10^3\ \mu^3$	$82.5 \times 10^3\ \mu^3$	$380.5 \times 10^3\ \mu^3$

During the late monaster- or streak-stage, measurements were made on the diameter of the hyaloplasmic envelope and on the diameter of the cell proper. Assuming that the shapes of both are spherical, the volume of the hyaline layer can be

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FIGURES 1 to 11. Photographs of successive stages of cleavage in *Strongylocentrotus pulcherrimus* showing the change in the thickness of the interspace between the hyaline plasma layer and the cell surface. Explanations are given in the text.

obtained by subtracting the volume of the cell proper from the volume including the hyaline layer, since there is no accumulated fluid at this stage.

If the same procedure is repeated just prior to the first cleavage when the interspace is wide, the volume of the hyaline layer plus the accumulated fluid can be obtained. Then, by subtraction, the volume of the accumulated fluid can be calculated. This calculation was applied to the four concentrations of gum arabic already mentioned. The results are given in Table I.

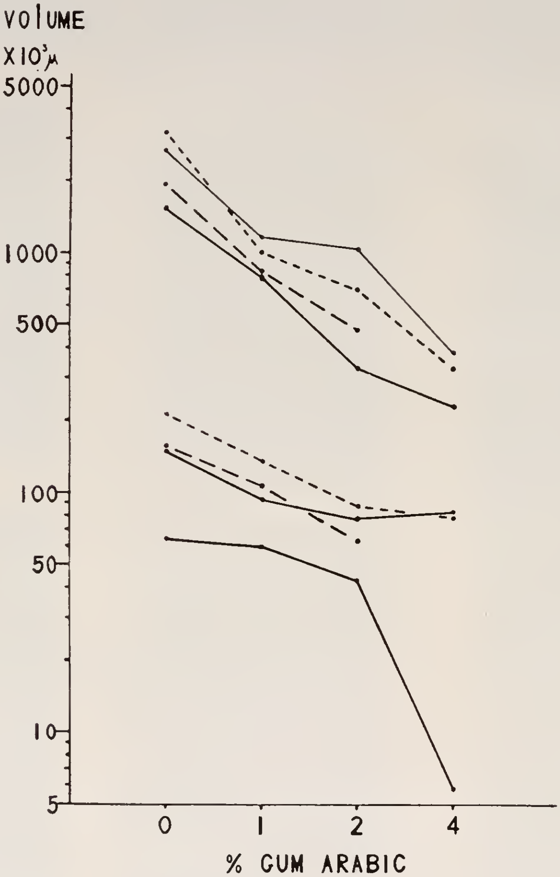


FIGURE 12. Correlated changes between the volume of accumulated fluid at one-cell stage (lower group of curves) and the volume of the segmentation cavity at the fourth hour after fertilization (upper group of curves) in different concentrations of gum arabic in sea water. Material: *Mespilia globulus.* Ordinates: volume of the fluid plotted on a logarithmic scale. Abscissae: concentration of gum arabic in sea water. Larvae were killed in a very dilute solution of OsO_4.

For the calculation of the volume of the blastocoelic fluid, morulae were used. In *Mespilia globulus,* a relatively large-sized segmentation cavity is formed in the early morula stage. At this stage, since practically all the fluid is in the segmentation cavity, and, moreover, since the shape of the cavity is roughly spherical, the calculation is very simple. The measurements at the fourth hour after fertilization are given in Table 1 and represented in Figure 12. There is an obvious difference, common to both the one-cell stage eggs and the morulae, between the volumes of fluid accumulated in the four concentrations of gum arabic. Moreover, a tendency can be perceived for lots of eggs which accumulate a larger quantity of fluid at the time of the first cleavage to develop into larvae with larger segmentation cavities. (Compare the highest and the lowest curves of Figure 12.) The proportionality between the fluid accumulation and the concentrations of gum arabic can also be seen qualitatively in the two-cell stage of *Mespilia* shown in Figure 13.

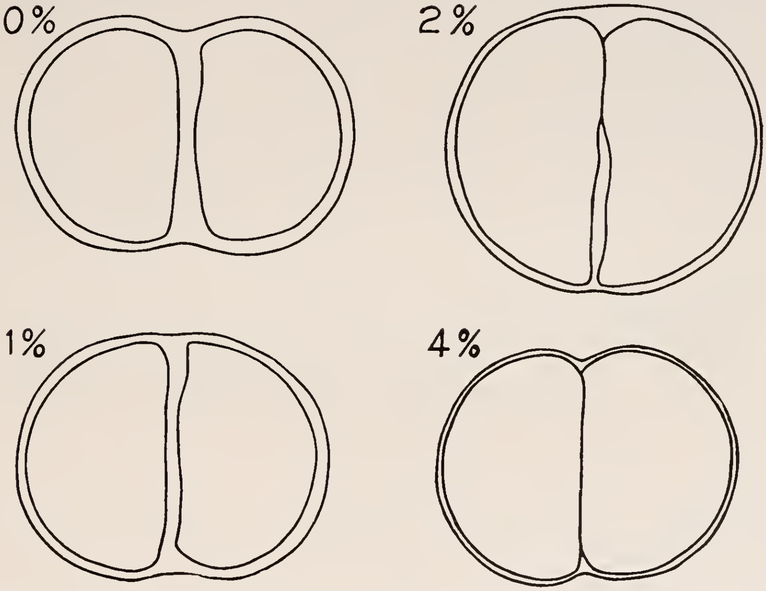


FIGURE 13. Illustrations of qualitative differences in the amount of the intra-hyaloplasmic fluid in different concentrations of gum arabic. The material is the two-cell stage of *Mespilia globulus*.

A different way to control the amount of the accumulating fluid and consequently the size of the segmentation cavity is to harden the hyaloplasmic gel so that it resists the entrance of the fluid. This is achieved by increasing the Ca concentration of the medium, according to the procedure described below. One-third molar $CaCl_2$, which is practically isotonic with sea water, was taken as the stock solution. This was mixed with Ca-free artificial sea water in a 1 : 1 ratio. For convenience, this mixture is called ½ Ca solution. A portion of the ½ Ca solution was diluted with Ca-free solution in the ratio of 1 : 1, which gave ¼ Ca solution. By the same procedure, ⅛ and $\frac{1}{16}$ solutions were prepared.

The eggs of *Strongylocentrotus* were fertilized and their fertilization membranes removed by shaking. One hour after fertilization, the eggs were transferred into the solutions of graded Ca concentrations and left in them until after hatching. In the pure isotonic Ca solution, the development ceased, while in other solutions, the development proceeded at practically the same rate as the control in sea water. After 21 hours, the larvae were anaesthetized and their contours and

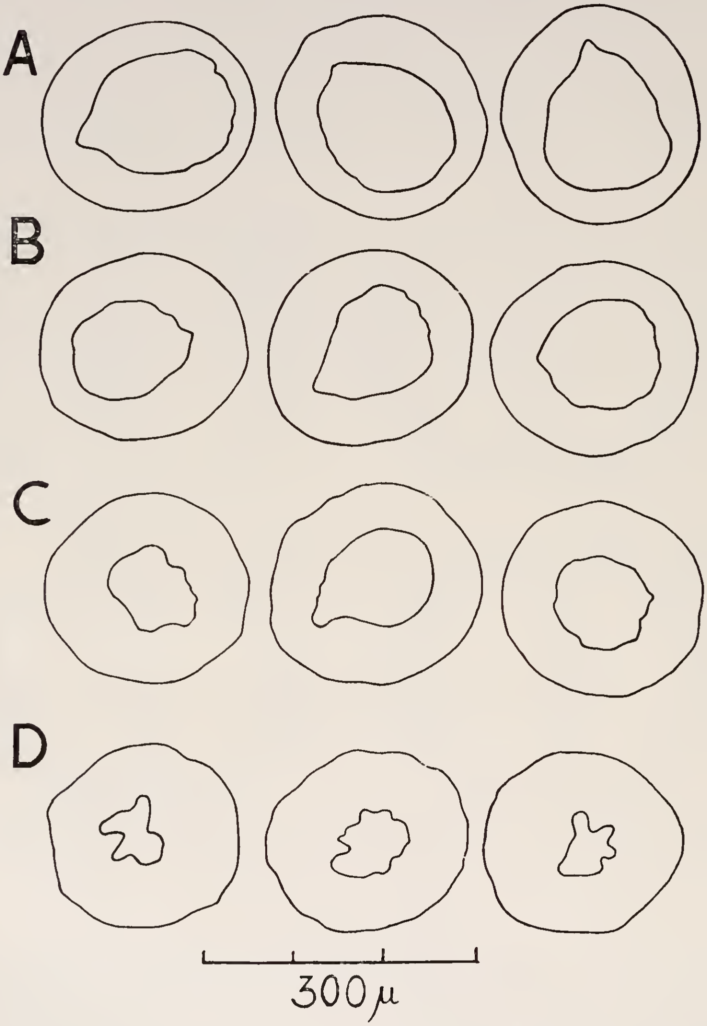


FIGURE 14. Twenty-one hour old morulae of *Strongylocentrotus pulcherrimus* reared in graded calcium concentrations. A. Control in sea water. B. $\frac{1}{3}$ *M* $CaCl_2$ 1 part + sea water 7 parts. C. $\frac{1}{3}$ *M* $CaCl_2$ 1 part + sea water 3 parts. D. $\frac{1}{3}$ *M*. $CaCl_2$ 1 part + sea water 1 part. Larvae were anaesthetised in chloretone when sketched.

the shape of the blastocoels were sketched, using a camera lucida. Some of the sketches are shown in Figure 14. The size of the blastocoel becomes smaller in proportion to the concentration of Ca present. The sketch of eggs in $\frac{1}{16}$ solution is omitted since scarcely any difference can be noticed from the control. Although this series of experiments cannot exclude the possibility that Ca ions are toxic and inhibit the development of the blastocoel in proportion to their concentration, taking the results of gum arabic experiments into consideration, it is highly probable that the hardened hyaline layer is suppressing the expansion of the blastocoel by its heightened rigidity.

From the foregoing facts, it can be inferred that the blastocoelic fluid is mainly made up of sea water to which a certain amount of colloid is added. The colloid is supplied at each cleavage through seven cleavage cycles in *Mespilia.* This idea is in harmony with the finding of Chambers and Pollack (1927) that the blastocoelic fluid of sea urchin and sand dollar larvae has a pH identical with that of sea water.

On the other hand, Monné *et al.* (1950, 1951) are of the opinion that the segmentation cavity of a sea urchin blastula is filled with a mucopolysaccharide gel which is secreted by the blastomeres, and the enlargement of the blastocoel is due partly to addition of more gel and partly to an increase in its swelling. The swelling of the gel is probably necessary to account for the large volume which the blastocoel eventually attains. But at the same time, it must be pointed out that the blastocoelic volume can be controlled by adding cane sugar to the surrounding sea water, as will be shown later, a fact which must be explained on osmotic grounds. If the blastocoel is filled with a jelly, the modification of its volume by sugar presents a rather complicated if not altogether insoluble problem. One thing which is clear from the present observations is that whether or not the substance in the blastocoel ultimately sets as a gel, it must be quite fluid at least in the beginning, since it flows from one place to another during the first few cleavage cycles. It may also be mentioned in passing that the blastocoelic substance of amphibian larvae can be collected by a fine capillary pipette (Stableford, 1949).

Hatching

In *Mespilia,* about the time of the tenth cleavage cycle, the larva begins to secrete the hatching enzyme; each blastomere acquires a cilium and the larvae begin to rotate within the fertilization membranes. The cilia penetrate the hyaline plasma layer and extend into the perivitelline space. Although it is extremely difficult to see the attachment fibers at this stage, it is presumed that the blastomeres still retain them. Within a short time after the larvae begin to move, the fertilization membrane is dissolved by an enzyme which they secrete, and the blastulae "hatch out" (Ishida, 1936). It may be worth mentioning that the above-described condition, *i.e.*, the fixation of ciliated cells to an enveloping membrane by attachment fibers, is closely simulated by *Haematococcus pluvialis,* a unicellular alga, with only the minor difference that the latter cell has two cilia.

Moore (1940) reported a conspicuous change in the nature of the blastular wall at the time of hatching. At this time the wall of the morula, which has been freely permeable to cane sugar molecules, suddenly becomes impermeable to them. This is shown by the fact that if an isotonic solution of sucrose is mixed with the medium, the shape of the morulae remains unaffected, while the swimming blastulae are deflated. Since the total osmotic pressure of the sugar mixture is the same as that of pure sea water, such deflation of the blastulae can only be explained by a selective impermeability of the blastular wall to the sugar molecules. Conversely, if

TABLE II

The volume of the fluid space under various conditions and various times

Sea water culture (SW) Control		Sea water—sugar (SW-CS)	
Time after fert.	Volume	Time after fert.	Volume
50–60 mins.	66.1 × 10^3 μ^3	—	—
3 hrs.	2061.9 × 10^3 μ^3	3 hrs. 30 mins.	2764.3 × 10^3 μ^3
4 hrs.	2874.9 × 10^3 μ^3	*4 hrs. 20 mins.	2807.0 × 10^3 μ^3
		4 hrs. 40 mins.	3294.7 × 10^3 μ^3 (morulae)
			2218.5 × 10^3 μ^3 (blastulae)
5 hrs.	4227.8 × 10^3 μ^3	5 hrs. 30 mins.	1237.8 × 10^3 μ^3
7 hrs.	3569.7 × 10^3 μ^3	7 hrs. 30 mins.	1052.7 × 10^3 μ^3

Sugar culture (CS) Control		Sugar—sea water (CS-SW)	
Time after fert.	Volume	Time after fert.	Volume
50–60 mins.	198.8 × 10^3 μ^3	—	—
3 hrs.	2665.1 × 10^3 μ^3	3 hrs. 30 mins.	3199.3 × 10^3 μ^3
4 hrs.	3381.1 × 10^3 μ^3	*4 hrs. 25 mins.	3592.4 × 10^3 μ^3
		4 hrs. 45 mins.	3955.7 × 10^3 μ^3 (morulae)
			10036.5 × 10^3 μ^3 (blastulae)
5 hrs.	4304.1 × 10^3 μ^3	5 hrs. 45 mins.	8623.3 × 10^3 μ^3
7 hrs.	4139.9 × 10^3 μ^3	7 hrs. 45 mins.	8124.8 × 10^3 μ^3

* Omitted from figures.

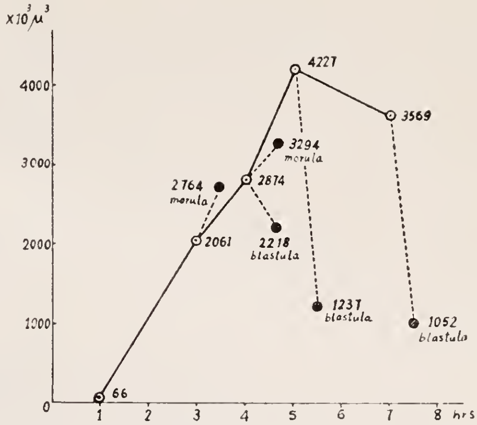


FIGURE 15. Effect of the transfer of *Mespilia* larvae from pure sea water into sea water + sucrose. ○: blastocoelic volume in sea water. ●: the same volume after being placed in 1:2 mixture of isotonic sucrose solution and sea water. No effect is found on the volume of the segmentation cavity of quiescent morulae while a large reduction in the volume takes place in swimming blastulae. Note especially the measurements at the fourth hour when hatched and unhatched larvae coexist.

larvae cultured in a sugar-containing medium until after hatching are returned to sea water, they swell because of a high internal pressure which is caused by the sugar molecules entrapped within the blastocoel.

Moore's experiment was repeated and the result re-examined from the author's standpoint. The procedure adopted was as follows: eggs were fertilized and the fertilization membranes removed mechanically. After the hyaline plasma layer had fully formed, the eggs were divided into two batches. One batch was transferred to a medium containing cane sugar solution (culture CS) and the other was left in sea water (culture SW). Pure cane sugar solution (isotonic) cannot be used, since the lack of Ca ions in this solution dissolves the hyaline layer. In a 1 : 2 mixture of sugar solution and sea water, the integrity of the hyaline layer is just maintained. Both 1 : 2 and 1 : 3 mixtures were tested.

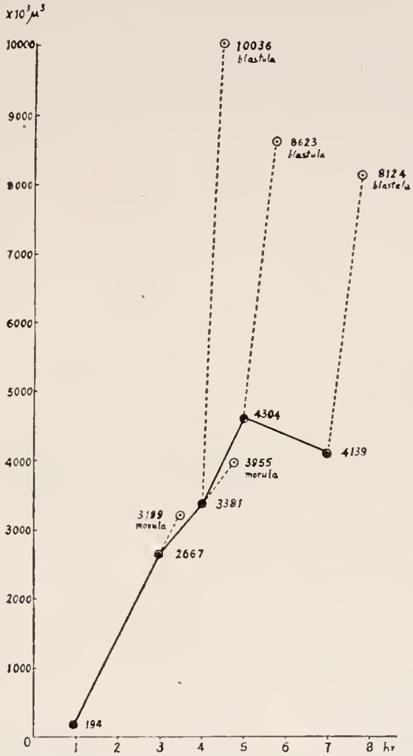


FIGURE 16. Reverse experiment to that shown in Figure 15. The *Mespilia* larvae are transferred from 1 : 2 mixture of isotonic sucrose solution-sea water into pure sea water. ○ : blastocoelic volume in sea water. ● : the same in 1 : 2 mixture of sucrose and sea water.

The diameter of the hyaloplasmic envelope was measured in the experimental lots and the controls during the streak stage and just before the first cleavage. The difference between the two sets of measurements gives the amount of the accumulated fluid in the cane sugar culture (CS) and that in sea water (SW). As the larvae reached the morula stage, the two batches (CS) and (SW) were further divided into four groups. The main portion of (CS) was placed in a fresh solution of (CS). For convenience in future comparison, this culture is labeled (CS-CS), although this is essentially (CS). A portion of the original (CS) was returned to pure sea water. This is called (CS-SW). Next, of the sea water culture (SW), a portion was changed to the sugar-containing medium (SW-CS), while the rest of it was transferred to fresh sea water (SW-SW). After half an hour of equilibration, the diameters of the segmentation cavities of the morulae were measured in the four solutions and compared. After an interval of one hour, the same procedure was repeated. The four new cultures thus obtained are designated $(CS\text{-}CS)_2$, $(CS\text{-}SW)_2$, $(SW\text{-}CS)_2$ and $(SW\text{-}SW)_2$. A third transfer at the third hour gave $(CS\text{-}CS)_3$, $(CS\text{-}SW)_3$, etc., and this procedure was continued until well after hatching. For making measurements, the larvae were killed by adding OsO_4 to the medium. The results of the calculation are given in Table II

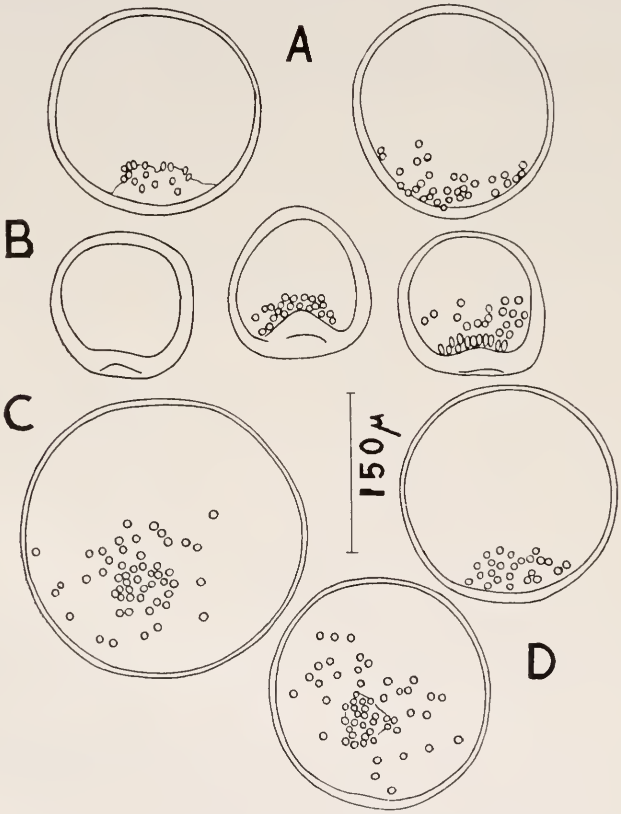


FIGURE 17. Sketches of *Mespilia* blastulae of a later stage in sea water and in sucrose-containing solution. The larvae were fixed with a small amount of OsO_4 added to the media. A. Larvae reared in sea water (series SW-SW) at 8½ hours after fertilization. B. The same batch transferred to sea water containing sucrose (series SW-CS) at 7 hours, and sketched at 9 hours after fertilization. C. Larva reared in sucrose-sea water mixture for 7 hours and then put in pure sea water (series CS-SW). Sketched 2 hours later. D. Larvae left in sucrose sea water for 8½ hours (series CS-CS).

and Figures 15 and 16, and sketches are shown in Figure 17 of later stages where a simple calculation is impossible.

From this experiment, it is clear that sugar does not suppress the accumulation of the fluid at the one-cell stage, in contrast to gum arabic. In the 1 : 2 mixture of sugar and sea water (CS), the volume was sometimes even larger than that in the sea water culture (SW), a result which can be interpreted as due to a softening or a swelling of the hyaline layer induced by the sugar. But certainly no evidence can be found indicating a deflating effect of the sugar on any part of the larvae. As for the change in permeability between the morula and blastula stages, Moore's findings were confirmed.

This change in permeability seems to take place within a relatively short time, as is shown in the following observations. If the *Mespilia* larvae which have been reared in the sugar-containing medium (CS) are transferred to pure sea water (CS-SW) at about the tenth cleavage cycle when they are just hatching, those which have already hatched and are swimming around swell to an enormous size. If all the swimming larvae are removed, and the culture left to stand for a while, a second lot of larvae hatch and begin to swim about. Such larvae of the second lot are of a medium size, while the third lot which hatch after the removal of the second lot are the smallest in size, that is, it is of the same size as controls left either in sugar mixture or sea water all the time. In other words, the size of the blastula is dependent upon how soon they hatch after the transfer from the sugar-containing medium to pure sea water; or, expressed differently, upon how much sugar is entrapped within the segmentation cavity when "the door closes" at hatching. Judging from the fact that the size difference is evident among batches collected 10 to 15 minutes apart, the process of permeability change probably does not require much time.

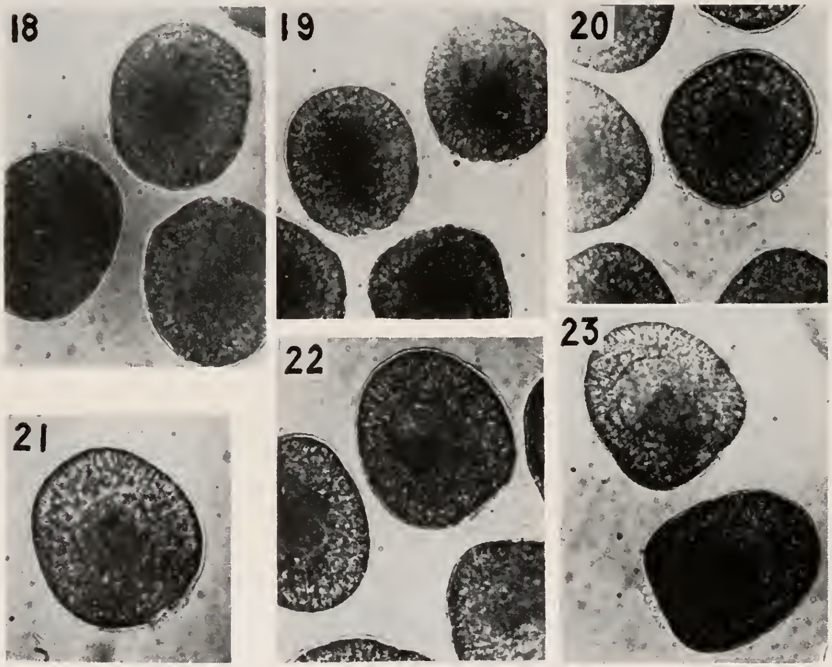
The determination of exactly what part of the larval system has thus changed its properties presents a problem. Since the cells themselves remain continuously impermeable to sugar, there are only two conceivable locations. One is the intercellular space and the other is the hyaline plasma layer. The latter statement may sound somewhat contradictory since it was previously stated that this layer was permeable to sugar during the morula stage. It must be recalled, however, that the hatching enzyme has been secreted in the meantime (Ishida, 1936; Sugawara, 1943). Under the influence of the enzyme, the nature of the hyaline layer might be changed in such a way that the passage of the sugar molecules is prevented. This possibility was tested by adding the enzyme to young morulae of *Strongylocentrotus* to test whether sugar impermeability could be induced. The negative result would indicate logically that the newly developed block to the passage of sugar molecules must be located in the intercellular space.

In *Heliocidaris crassispina,* the segmentation cavity normally does not develop as extensively as in other forms, while the larva remains within the fertilization membrane. But as soon as it hatches, the size of the cavity increases rather abruptly by expanding in an antero-posterior direction (see Plate II). As this elongation takes place, the thickness of the anterior blastula wall decreases, suggesting that the elongation is allowed by the expansion of the anterior, or animal pole region of the larva.

In *Mespilia globulus* and *Clypeaster japonicus,* which form spacious segmentation cavities, the larvae are spherical while they remain within the fertilization membrane. However, when they elongate along the antero-posterior axis after hatching, a similar thinning of the anterior wall, as in *Heliocidaris,* can be observed. But whether this thinning is a passive stretching or an active expansion is a point to be settled in the future.

The volume changes occurring when the blastulae are put into or taken out of sugar mixtures were followed until the seventh hour after fertilization, and the resulting data are included in Figures 15 and 16 and Table II. After this stage, although calculation of the volume becomes difficult, similar inflation and deflation of the blastocoel can be noticed qualitatively, as is illustrated in Figure 17.

PLATE II



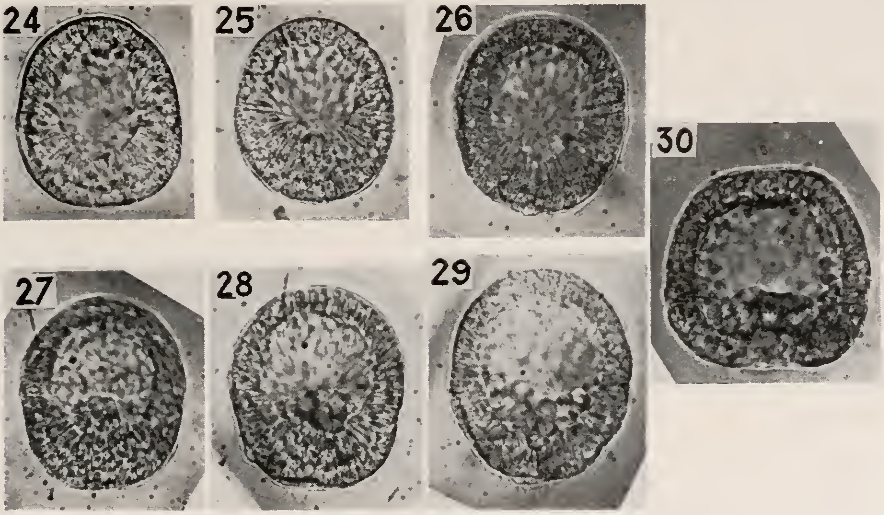
FIGURES 18 to 23. Consecutive stages of the blastulae of *Heliocidaris crassispina* showing gradual lifting of the hyaline layer at the vegetal pole. Larvae were fixed by adding a small quantity of OsO_4 and photographed immediately.

In connection with these three figures (15, 16, 17), there are two points which should not be overlooked. (1) The data of the seventh hour clearly indicate a spontaneous decrease in the blastocoelic volume of the larvae cultured in sea water (SW) as well as in sugar mixture (CS) (see Figures 15 and 16). (2) One hour and a half later, the deflation of the 8½ hour larvae by the sugar mixture (SW-CS) can actually cause a precocious gastrulation. There is no room for doubt that the indentations shown in the larvae of the B-group are really invaginations because they invariably occur at the site of the future blastopore. At this stage, the orientation of the larvae can very easily be determined both by the direction of their swimming and by the position of the primary mesenchyme cells. This point will be pursued in future investigations.

Mesenchyme Formation

Another stage of some interest is that in which the primary mesenchyme cells are formed. As is well known, these cells are derived from the vegetal wall of the blastula and liberated into the segmentation cavity. If the blastomeres are attached to the hyaline plasma layer, as is proposed in this paper, they must first break this connection in order to wander freely into the blastocoel. This would very likely affect the overlying hyaline layer in some way or other. With this expectation in mind, particular attention was paid to the vegetal region of the blastula of several species of available sea urchins. An indication was promptly discovered.

PLATE III



Figures 24 to 30. Figures 24–29 show a relation between the migration of the mesenchyme cells and the lifting of the vegetal hyaline layer in *Strongylocentrotus pulcherrimus*. Figure 30 shows the condition after gastrulation. Larvae were fixed in dilute OsO_4 solution.

Roughly speaking, the changes observed in the hyaline plasma layer of the vegetal region can be divided into two categories. One type is that of *Clypeaster* larvae, in which the vegetal hyaline layer seems to disintegrate or dissolve. If examined carefully, vaguely perceptible, thread-like objects, to which various particles are often stuck, can be seen at the vegetal end.

The other type, which includes all the regular sea urchins so far examined (*Mespilia, Heliocidaris, Pseudocentrotus, Strongylocentrotus*), is characterized by the formation of blister-like lobes of the hyaline layer, which become larger as development advances. (See Plates II and III.) The cause for this seems to be understandable. When a mesenchyme cell frees itself from the blastular wall, the space which has been occupied by the cell is closed in by the surrounding cells.

Thus the portion of the hyaline layer which has been in contact with the mesenchyme cell loses its cellular support and will be pushed up as a folding or a pocket. As the number of the wandering cells increases, the pockets will fuse into a larger blister. This is, at least, the impression the author received from *Heliocidaris.* Interestingly enough, Runnström (1935) describes structures like this on the vegetal side of *Echinarachnius parma* larvae, although he does not venture any opinion on the nature of the structures.

Discussion

On the basis of these various observations, the following description of early sea-urchin development is proposed. Following the fertilization of the egg, the hyaline layer is secreted and the attachment fibers are formed, binding the cell surface to the hyaline layer. Once this attachment is established and cleavage proceeds, the only additional condition necessary for production of a morula is inflation of the hyaline envelope. This occurs at various stages in the various species, but mostly in a stepwise fashion, in connection with the division activities. As long as the envelope grows larger, regardless of how and when this result is achieved, in expanding the envelope will carry the cells to their final position. In this sense, the hyaline layer has a significance somewhat greater than that of a simple basement membrane, since it leads the movement of the cells in the formation of the embryo.

This method is very well adapted while the larva is increasing its volume at a rapid rate within the fertilization membrane, since there cannot be any time lag. However, after the larva hatches out, it may not be a very reliable one since a slight damage in the hyaline gel will make the larva collapse and shrivel.

The sudden onset of a closer association among the cells, as indicated by the sugar impermeability, seems to work so as to put a brake on this fragile system and make it set to a certain size.

The author has not been successful in discovering the nature of the change occurring in the intercellular space of the blastula. However, it is extremely interesting that this close association among the cells is not only correlated with their acquisition of the capacity to secrete the hatching enzyme—it is also correlated with the beginning of the conduction of impulses controlling the ciliary beat. The change in physiological state which the larvae undergo during this short interval while hatching takes place is tremendous—the blastomeres of the morula stage, which have been only individually fastened to a common envelope, suddenly change into tissue cells. This new, close association among the cells would certainly fix the shape of the blastula.

Now, if one imagines that some decrease in the blastocoelic volume occurs, either as a result of disintegration of the colloid or shrinkage of the gel, the newly acquired tissue rigidity will no longer allow the larva to shrink evenly to a smaller sphere, but will cause indentations in the blastular wall, as in a rubber ball from which the air is extracted. The author is tempted to think that such a mechanism plays at least a partial role in the process of sea urchin gastrulation. A few facts indicating this possibility have been pointed out in connection with Figures 16, 17 and 18. Future investigations will be conducted in this direction.

SUMMARY

1. The accumulation of a fluid under the hyaline plasma layer of *Strongylocentrotus pulcherrimus* eggs, and its movement during cleavage and interkinesis are described. It is shown that an increase in volume takes place in connection with each cleavage, and that the accumulated fluid comes around to the center of the larva, giving rise to the segmentation cavity.

2. Gum arabic prevents the fluid accumulation during early cleavage periods and reduces the volume of the segmentation cavity in later stages. Raising the Ca level of the medium brings about a similar result, by causing a hardening of the hyaline plasma layer and thus preventing increase in size.

3. Moore's finding—that at the time of hatching, the blastular wall, which has been permeable to cane sugar molecules, suddenly becomes impermeable to them—is confirmed.

4. After the migration of the primary mesenchyme cells from the blastular wall into the blastocoel, the vegetal hyaline layer which has been in contact with these cells is lifted up as folds in regular sea urchins. In the larvae of irregular sea urchins, it is dissolved away.

5. The possible significance of changes in the mode of association of the component cells on certain gross morphological changes of the sea urchin larva is discussed.

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STUDIES ON THE PIGMENTATION CHANGES DURING RECONSTITUTION IN TUBULARIA

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A noteworthy feature during reconstitution of the marine hydrozoan Tubularia is the appearance of a reddening at the site of hydranth formation. This apparent concentration of pigment suggests a possible relation of the pigment to the metabolic processes of reconstitution.

Moreover, studies on the mechanism of dominance in this form have suggested to Barth (1938) that there is a competition by the ends of a stem piece for something in the circulation. When the circulation is blocked by oil drops (Barth, 1938), glass rods (Rose and Rose, 1941), or ligatures (Barth, 1938), dominance is obliterated. When an open capillary tube of short length is inserted into the coelenteron and the stem ligated about it, thus breaking the coenosarcal connection but temporarily keeping some fluid exchange, a dominance effect was noted by Rose and Rose (1941).

Since pigment granules are very evident in the circulation during regeneration, an investigation of the origin, fate and nature of the pigment was undertaken.

MATERIAL AND METHODS

The work reported here was done on Tubularia supplied by the Marine Biological Laboratory at Woods Hole during August, 1951. The stems were four to eight inches long and unbranched. The hydranths show the tentacle characteristics reported for *Tubularia crocea*. The coelenterons were usually partitioned, starting just below the hydranth constriction, by tissue sheets connecting the gastric ridges. These sheets may connect adjacent ridges, in which case the cross-section shows cords less than a diameter, or several sheets may seem to originate from a center giving a wheel-hub effect in cross-section (Fig. 1, C and D). The flagellated cells responsible for fluid circulation are mainly at the two junctions of any gastric ridge with the rest of the coenosarc as seen in cross-section.

The pigment of Tubularia is typically red. It has been long observed that this redness is variable but an unusual event, noticed by the Woods Hole Supply Department and by us for the first time in August, 1951, is that interspersed with the red stems were yellow and orange stems, as well as intergrades. These stems were in no apparent way otherwise different from the red ones. The colors occurred in both young and old hydranths, both sexes, and in stems of differing lengths. It was not unusual to find a yellow stem in the midst of red stems. These individuals offered a new approach to the problem of pigmentation during regeneration.

Unless otherwise noted, all stem fragments were 5 mm. in length and were taken from a point 3 mm. behind the hydranth constriction. To keep track of the original polarity, the distal cut is made horizontal and the proximal oblique. Where a group of four or less stems was being followed, the stems were placed in 50-ml. Erlenmeyer flasks half filled with sand-filtered sea water. Where 5 to 10 stems were being followed as a group, they were kept in fingerbowls in 100 ml. of filtered sea water. All containers were placed in an illuminated cold room at 16–18° C. The average time for regeneration of a hydranth was about three days.

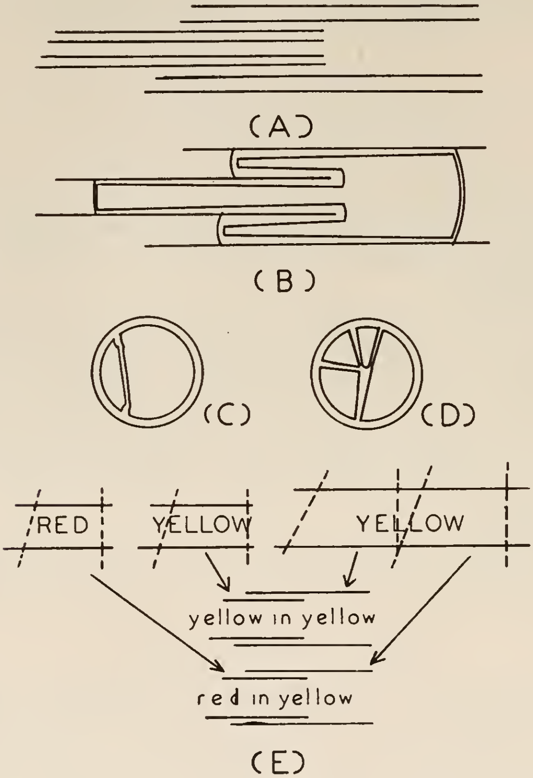

Figure 1. For explanation, see text.

Experimental

Preliminary experiments involving regeneration of sections from hydranths of colors other than red showed that without exception the regenerant was of the same color as the original. In some cases a decrease in color intensity seems to have occurred, but this is not clear cut. An experiment was then performed wherein portions of red stems were allowed to fuse with yellow stem fragments, to determine the influence of this situation on the color of the hydranth forming in the yellow component.

All yellow stems were taken from animals bearing yellow hydranths, and all red stems from animals bearing red hydranths. Hydranth and stem color always corresponded, although the intensity was both less and somewhat more variable in the stem portion.

For the fusion experiment, stems were selected which were either intensely red or pale yellow. For each set in this experiment, one yellow and one red stem of approximately equal diameter, and a second yellow stem of larger diameter were used (Fig. 1, E). Two 3 mm. fragments were cut from the larger stem and one each from the smaller stems. Under the binocular microscope, the distal end of the red piece was inserted into the proximal end of one of the large yellow pieces, and similarly the distal end of the small yellow fragment was inserted into the proximal end of the other large yellow piece. The original polarity was therefore maintained. Every effort was made to align the gastric ridges of the pieces, since preliminary experiments showed that failure to do so tended to prevent fusion of the fragments. It was considered preferable to insert the red stem into the yellow one rather than the converse, since it is possible that the hard perisarc of the insert might traumatize the coenosarc of the containing piece and liberate pigment. A few such converse experiments will be reported as controls. The use of the large yellow fragment as the containing piece also had the benefit of tending to make the hydranths form in the yellow component, since the fused 3 mm. fragments behave like a single 6 mm. stem piece in exhibiting dominance. The healing process is shown diagrammatically in Figure 1, A and B. One might largely insure formation of the hydranth in the yellow end by ligation of the red end, but ligation definitely damages tissue and so this was avoided. The colors of the regenerated hydranths were examined against a black background with strong white side lighting. The colors are unambiguous. The results are presented in Table I.

The significant results are those in which (1) successful fusion has occurred, with the coelenteron being common to both components, and (2) the formation of the hydranth has occurred in the yellow end of a red stem fused to a yellow stem. It will be noted that in every such case the hydranth was redder than the hydranth of the control consisting of two fused yellow pieces.

Observations of fused stems during the regeneration process show that the red pigment appears in the circulation following healing of the cut ends. It then seemingly is picked up by the gastric ridges. The gastric ridges of the yellow components can be seen with lines of red particles. This effect varies in uniformity since the gastric ridges do not occur symmetrically and the circulation currents vary in rate of flow and may have eddies. As noted by Stevens (1901) there is a tendency for the gastric ridges to break down as regeneration proceeds and the picked-up pigment tends to be re-liberated. When the primordia of the tentacles form, however, it can be inferred that pigment particles are picked up by them, as chains of these particles mark the tentacular ridges. There are also some randomly distributed particles in this area.

The above experiment was repeated in a few cases with oil drops placed in the coelenteron of the yellow components before healing occurred. These drops block the circulation. In these cases the hydranth color was the same as that of the control hydranth. This might also be held to rule out a factor transmitted by the red component to the yellow via the coenosarc. The oil drop, however, may affect the coenosarc adjacent to it by limiting the entering and leaving of metabolically important materials, and thus effectively also block the coenosarc pathway.

TABLE I

A: Success of Fusions
All fusions successful and normal except:

Set No.	Yellow to Yellow	Yellow to Red
7	S	U
11	U, cytolyzed	U, abnormal
12	U	S
15	S, abnormal	S
26	U	S, abnormal

B: Observed Color of Hydranths

Fusion:	Yellow in Yellow	Red in Yellow		Yellow in Red	
Hydranth-bearing end:	Either	Red	Yellow	Yellow	Red
Set No.					
1	Y	O	O		
2	Y	R			
3	O		OR		
4	O				OR
5	Y		O		
6	Y			Y	
7	O	R	Y		
8	YO	OR			
9	Y		O		
10	YO			OR	
11	—	O			
12	Y			OR	OR
13	Y	R			
14	YO		OR		
15	Y		O		
16	Y		O		
17	Y		OR		
18	Y		OR		
19	Y		O		
20	Y	OR			
21	Y		O		
22	YO		OR		
23	Y	OR			
24	Y	O	O		
25	Y	OR	O		
26	Y	OR			

KEY: S—successful; U—unsuccessful; Y—yellow; YO—yellow-orange; O—orange; OR—orange-red; R—Red.

Since one could not see individual particles being picked up, the possibility also remained that some soluble pigment precursor was being produced by the red stem and entering the yellow. Attempts to insert filters of various sorts into the coelenteron failed and the procedure was intrinsically undesirable, as it was impossible to place these without severe damage to the gastric ridges and partitions.

A probable resolution of these questions was effected in the following manner. "Norite" animal charcoal was ground in sea water with a mortar and pestle and a suspension of fine particles injected with a micropipette into the coelenteron of various stem pieces. These particles tend to adhere to the gastric ridges. Following healing, the particles become loose with breakdown of the ridge and are readily seen in circulation. This might simply be a washing free as circulation begins, but the disappearance of spot stains of Nile blue sulphate suggested an actual breakdown. With regeneration the charcoal particles tend to adhere in the area of hydranth formation and particle chains delineate the primordia of the tentacles. In one case where a stolon started to form, a tendency was noted for the charcoal particles to adhere at this area. In every respect the distribution of red pigment particles and charcoal particles is the same.

Smears of emerged hydranths following charcoal treatment show that the pigment masses and charcoal are similarly localized in the endoderm and between the endoderm and ectoderm layers. Sections of forming and emerged hydranths, following Zenker's fixation and staining with carefully filtered Heidenhain's haematoxylin without counterstain, confirm the charcoal distribution as observed in smears. Small charcoal fragments appear to be inside some endoderm cells. No mitoses were evident in the regenerating areas.

The above observations are regarded as making it highly probable that pigment particles are actually picked up. It was then decided to examine the character of the circulating fluid.

Stevens (1901) reports the contents of the circulation as pigment granules, translucent globules and nuclei. Hargitt (1903) records the presence in the same species of cnidocysts in the endoderm and circulation. We have examined the character of the fluid withdrawn from circulation with a fine pipette and confirm the presence of cnidocysts, pigment granules and cell debris. The material in circulation varies according to the time after healing when the fluid is examined. This may account for the failure of Stevens to find cnidocysts.

To determine the origin of the circulating material, smears of stem tissue were examined for obviously specialized cell types. In the endoderm were seen numerous cnidocyst cells and round or oval pigment cells. The latter are generally yellow but frequently have in them darker orange or red masses suggesting storage or concentration of this material.

In the course of regeneration, the area at an end where a hydranth is not forming becomes transparent; it was therefore decided to check to see whether this was an actual thinning or simply a loss of opaque material such as pigment. An actual thinning might be due to local cytolysis or a contraction of tissue into other areas, or to cell migrations. Therefore, sections of stems were cut and immediately thereafter additional cylinders approximately 1 mm. in length were cut from the remaining stem just proximal and just distal to the 5 mm. fragment. Working rapidly, these were immediately stood on end and the tissue thickness measured, using an ocular micrometer. The magnification was 43 × and 1 mm. of tissue was 10 micrometer units. This procedure is not adequate for small changes; however, it was readily observed that changes of the magnitude of a 50% decrease in coenosarc thickness occurred when, during regeneration, 1 mm. cylinders were cut from the stem piece up to the area of hydranth formation. It was not possible to judge with certainty whether a gradient of thickness, showing increase in the direction of the hydranth, occurred although this is the visual impression. The need for speed in measurement is due to the fact that with the onset of the healing process the cut ends curl inward and increase in optical cross-section. The hydranth-forming area, as expected, becomes very thick and almost obliterates the coelenteron. Measurements were made on perisarc thickness concurrently as a check on technique, and these showed no change.

Chemical studies of a preliminary nature were then made on red stems, only, in an effort to obtain some information about the pigment. The pigment proved to be water insoluble but soluble in organic solvents, particularly polar solvents such as acetone, methanol and pyridine. With concentrated sulfuric acid a blue color is obtained. A chloroform solution gives a positive Carr-Price test for carotenoid by giving a blue-green color. Absorption measurements were made on various extracts using a Beckman spectrophotometer. In every case, single peaks in the visible range were obtained. These were: 490–500 mμ in pyridine; 455–70 mμ in petrol ether; and 470–80 mμ in acetone. The only carotenoid listed in Karrer and Jucker (1950) showing such characteristics is astacene. Astacene's solubility properties are very similar to those of the red pigment. Astacene is a pigment occurring mainly in animals, only one case being known of its presence in a plant, an alga. It is, however, supposedly an extraction artefact of astaxanthin and results from oxidation during extraction. Attempts to obtain astaxanthin by homogenizing hydranths in acetone-solid CO_2 slush and extracting under nitrogen failed but are not regarded as being final.

The above extracts were made on hydranths only, since numerous red algae and other organisms grow on the perisarc. It was noted, however, during the course of tissue smears, that our material was frequently infected with red algae in the endoderm. The most common of these resembles *Ceramium*.

As a preliminary to future work, an extract of hydranths of all colors in petrol ether was chromatogrammed on a sucrose column. Three red bands, one yellow band and one orange band were noted, but no attempt was made to collect the fractions. The bands may be due to different chemical chromatophores, to different esterified combinations of the same chromatophore, or to natural or extraction-created isomers.

Discussion

Loeb (1891), who first noted the color changes in regeneration, suggested that the pigment might be nutritive. Driesch (1900), noting that the pigment particles seemed to be linearly arranged following the tentacular primordia, believed that they might have morphogenetic significance. Morgan (1901) challenged this, however, on the basis that despite evident considerable variation in pigmentation, regeneration proceeded normally. He also noted that the stolon frequently showed the pigment.

There is still no evidence of any functional significance for the pigment of Tubularia. The non-random arrangement of pigment particles seen by Driesch as delineating the tentacular primordia is now seen to be due to a mechanism which indiscriminately takes up particles. The presence of cnidocysts in the endoderm and in circulation suggests that this may be the normal method of supplying the cnidocyst units of the new hydranth. The presence of charcoal particles between the two layers but not in the ectoderm would then suggest that the mechanism is particle-indiscriminate up to this point, but that incorporation into the ectoderm involves discrimination.

Against this concept it must be noted that charcoal and pigment tend to adhere where the stolon begins to form. This may mean that these growing areas simply have sticky surfaces and that the lining-up of particles in the tentacular primordia is the incidental result of cell movements.

Stevens (1901) and Godlewski (1904) believe that the red pigment is liberated by endoderm cytolysis, particularly of the gastric ridges, during regeneration, and is a waste material expelled when regeneration is complete. We confirm the liberation of some pigment with gastric ridge breakdown.

The pigment may indeed be a waste product derived from ingested crustaceans who, in all probability, contain astacene (or astaxanthin). There is no denial that some is frequently expelled after reconstitution. Preliminary chromatography does suggest, however, that it may pass through several chemical stages in Tubularia, as is also suggested by the occurrence of strikingly different colored specimens in the same clump of stems. These could be biochemical mutants.

The storage of pigment in Tubularia might serve as a prey-attracting mechanism. No evidence exists on this point. Carotenoid pigments are of known importance in visual biochemistry. There is no evidence on light sensitivity in this form. Carotenoid materials have been reported as gamete-attracting agents (Moewus, 1940). While this has not been confirmed, the pigment here occurs in both sexes and in apparently similar distribution.

Regarding the chemical nature of the pigment, Driesch (1900) remarks that it was identified for him as a carotenoid, and Lönnberg and Hellström (1931), working with *T. larynx,* name it as astacene. However, they report a main absorption band in petrol ether at 432–4 mμ and minor bands at 458–9 mμ and 488–91 mμ. Our work confirms their identification but not the presence of three bands. Our data are in agreement with the spectral and other characteristics described by Karrer and Jucker (1950) for astacene.

It is undisputed that the area of hydranth formation shows a prior reddening, and the latter must be due to one or more of the following events: 1) local synthesis of pigment; 2) local acquisition of pigment formed elsewhere; and 3) more pigmented cells entering the area. Therefore, histological studies of the cell movements, divisions and growth relative to regeneration are important. Histological studies have been made by Bickford (1894), Stevens (1901), Godlewski (1904) and Hargitt (1903). Alone among these, Stevens reports mitotic figures in the regenerating area, both in ectoderm and endoderm. She also noted that in her sections the tissue in the non-regenerating area had become thin and the cells elongated in the stem length. To her, this suggested contraction of cells into the regenerating area. Contraction would account for reddening by increasing the length of the optical path through the pigmented material. This is readily duplicated by ramming tissue in the perisarc with a glass rod. Hargitt, unlike Stevens, finds no mitoses and believes that amitotic divisions must occur. Cell divisions are necessary to account for the increase in ectoderm surface, according to Hargitt. Bickford's work is directed towards the successful establishment of the view that the hydranth must form from previously differentiated cells, rather than from a primitive cell, but she does not touch the problem of which differentiated cells form the hydranth. Against the contraction hypothesis of Stevens is the old observation of Allmann (1874) that no muscle cells exist in the tissue under the perisarc. This is not necessarily a valid objection since all cells are contractile to some degree.

Our work confirms the decrease in tissue thickness and the absence of distinguishable mitotic figures which therefore would lend weight to a cell movement theory. It must be pointed out, however, with regard to the observation regarding mitoses, that it is possible that we are dealing with mitoses of an unusual type. Mitoses might occur in waves, for example, and thus not be picked up in sections made on 5 or 6 stems. This would explain Stevens' finding them and the failure of the other observers to do so. Work with agents which arrest mitoses might clarify this point.

One still may not say that the reddening which marks the site of hydranth formation is entirely due to picked-up pigment. While this and intrinsic pigment could account for a good part of the reddening, contraction of tissue into the area is not ruled out. The discreteness of the particles marking the tentacular primordia, however, suggests that the former originate entirely from picked-up pigment.

Summary

1. At least part of the reddening designating the site of hydranth formation in Tubularia is due to pigment removed from circulation.
2. The mechanism removing pigment particles from circulation does not distinguish them from charcoal particles introduced into the stem. These become distributed in exact coincidence with the pigment in the reconstituted hydranth.
3. The extracted pigment of Tubularia seems to be mainly astacene, as judged from its spectral and solubility properties.
4. The immediate origin of the pigment in circulation is from the breakdown of endodermal tissue.
5. The possible significance of removal of pigment is discussed.
6. Color variants in Tubularia are noted, as well as infecting red algae.

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THE BIOLOGY OF THE SIERRA LUMINOUS MILLIPEDE, LUMINODESMUS SEQUOIAE, LOOMIS AND DAVENPORT

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In May of 1949 a group of students on an ecology field trip to the Sequoia National Forest (Camp Nelson, Tulare Co., California) brought back to the senior author a large number of striking and brilliantly luminescent millipedes. These animals were sent to Dr. E. Newton Harvey of Princeton University, who in turn forwarded them for identification to Mr. H. F. Loomis of the U. S. Plant Introduction Garden, Coconut Grove, Florida. Mr. Loomis found them to constitute an undescribed genus and species, and there resulted a collaborative original description (Loomis and Davenport, 1951). Reports of luminous millipedes are few. Bruner (1891) observed and described animals in Nebraska which were later given the name *Fontaria luminosa* by Kenyon (1893) and thus placed in the family Xystodesmidae, in which Luminodesmus belongs. It would appear, however, that these studies constitute the first investigations to be made of the life-history and physiology of luminescence of a luminous diplopod.

Luminodesmus sequoiae is a large and handsome millipede, the adults averaging 40 mm. in length and 8 mm. in width, of a pale tan or salmon color in late adult life, with a dark mid-dorsal line. In darkness the entire animal and its appendages give off a brilliant greenish-white glow, plainly visible to the dark-adapted eye at a considerable distance. Figure 1A is a photograph in ordinary (stroboscopic) illumination, while Figure 1B shows the animal taken in its own light only.

After discovery and description of the millipede, it became apparent that a thorough investigation of its ecology, distribution, life-history and physiology should be undertaken. Accordingly, in May of 1951 a trip was made to the type-locality for the purpose of collecting numbers of living animals, and some four hundred or more were brought back to Santa Barbara.

In the laboratory the millipedes proved easy to culture. A minimum of care was necessary to keep them alive and to raise the early stages. Adults were kept in a series of standard glass terraria filled to a depth of 5 or 6 inches with the rich, dark humus collected in their habitat. These terraria were covered with glass sheets and sealed with vaseline; about every 10 days they were opened and the humus

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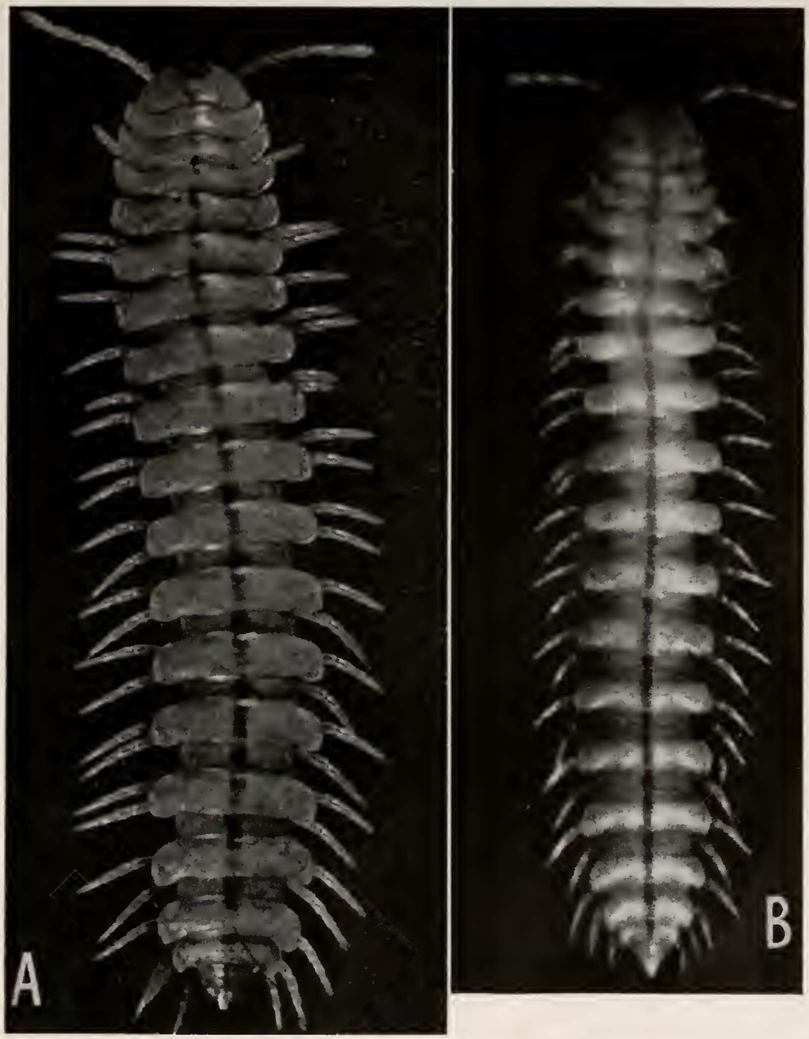

FIGURE 1. A. Adult *Luminodesmus sequoiae*. Approx. × 3. B. An adult *L. sequoiae* photographed in its own light. Animal curarized. Approx. × 3.

lightly sprinkled with water. Occasionally, when animals were removed for experimental purposes or a search for early stages was made, fresh humus was added from storage containers brought in from the field at the time the animals were collected.

ECOLOGY AND DISTRIBUTION

Luminodesmus sequoiae appears to have a very limited geographic range. So far the only records are one from the type-locality and an uncertain one from

Kaweah, Tulare Co. (Loomis and Davenport, 1951). During the summer of 1951 a brief visit was made to Yosemite, Kings Canyon and Sequoia National Parks during which it was found that no such animals had ever been observed in the parks to the knowledge of the Park Naturalists and their staffs. One ranger stated that he had observed "large luminescent animals" on a trail along the Merced River at low altitudes in Yosemite, but that these were not identified.

It appears probable that the animals may be limited to a particular environment with the characteristics of the type-locality, relatively moist with a thick, dark humus under a leafy litter made up of the disintegrating leaves of *Quercus, Acer,* etc., with an admixture of conifer needles. They must occur rarely if at all in the heavy coniferous forests of higher altitudes in the Sierra, characteristic of Kings Canyon and Sequoia National Parks; otherwise it would seem that they could scarcely have escaped notice. However, it is likely that further investigation of areas similar ecologically to the type-locality at lower altitudes within the Parks and in the foothills beyond their western limits may extend the range of Luminodesmus.

As has been stated, at the type-locality the animals may be so numerous on the surface that at night, when their bright patch of light attracts the eye, dozens can soon be collected. An effort was made in May of 1951 by Dr. E. R. Noble of the collecting group to determine how common the developmental stages might be in the upper layers of humus and soil. It was found that their distribution was extremely irregular. Several areas two feet square were carefully sorted over to a depth of 6 inches. In one such area 54 animals, ranging in length from 2–35 mm., were collected. Some areas of similar size had a far smaller population and in others no millipedes whatever could be found. No very striking differences in the characteristics of these areas could be observed which might account for the difference in distribution. It is apparent that the animals may be numerous enough locally so that their feeding activities could have a marked effect on the characteristics of the humus in their particular niche.

An investigation at the type-locality in October of 1951 showed that at this late season after a summer of extreme dryness adults were almost totally absent. A careful search on two consecutive nights revealed no adults at the surface. During daylight a number of areas where large numbers had been taken the previous spring were carefully investigated by digging to a depth of approximately 9 inches. It was found that although certain of these areas, particularly under the overhanging edges of logs and boulders, were filled with well defined workings and broken cocoons, no adults of the preceding summer were present. In some areas, however, numbers of the last four larval stages were discovered, seemingly distributed at random in the first 6 inches of soil, while a number of recently moulted adults were also present. All living animals were very inactive and there were indications of a high mortality due to desiccation, since a number of intact cocoons containing the dried remains of animals were found.

It would appear, therefore, that very few adult animals survive the long period of desiccation from mid-summer until the beginning of the winter rains, at least in years of light rainfall. This observation is borne out by the comment of one of the district foresters, stationed at Camp Nelson, who on being questioned, said that he was familiar with the animal and that it was a creature of the spring rains, disappearing at the beginning of the summer drought.

LIFE-HISTORY

Millipedes that were kept in humus in the sealed terraria in the laboratory thrived for many months, burrowing through the humus in their feeding and coming actively to the surface at night to wander about. The room in which they were kept varied in temperature in the usual way during the day from approximately 65° F. to 85° F. Because of the relatively constant temperature of this artificial environment and the knowledge that at least during several months of their development in nature they are subject to daily extremes of temperature from well below freezing to above 80° F., it did not seem worthwhile to go to the difficulty of determining in more than a general way the time-duration of each stage.

When the adult animals were first brought into the laboratory in May they were placed in fresh humus known to be free of eggs or early stages. Although mating was not observed in the laboratory, it is reasonable to assume that it freely occurred, since members of both sexes were placed in the terraria at random. The terraria were first investigated for the presence of early stages on July 19th and numbers of egg-masses were found (Fig. 2A). The date of laying was not known. These egg-masses varied widely in the number of contained eggs; three that were counted contained approximately 160, 70 and 165 respectively. The masses were laid in

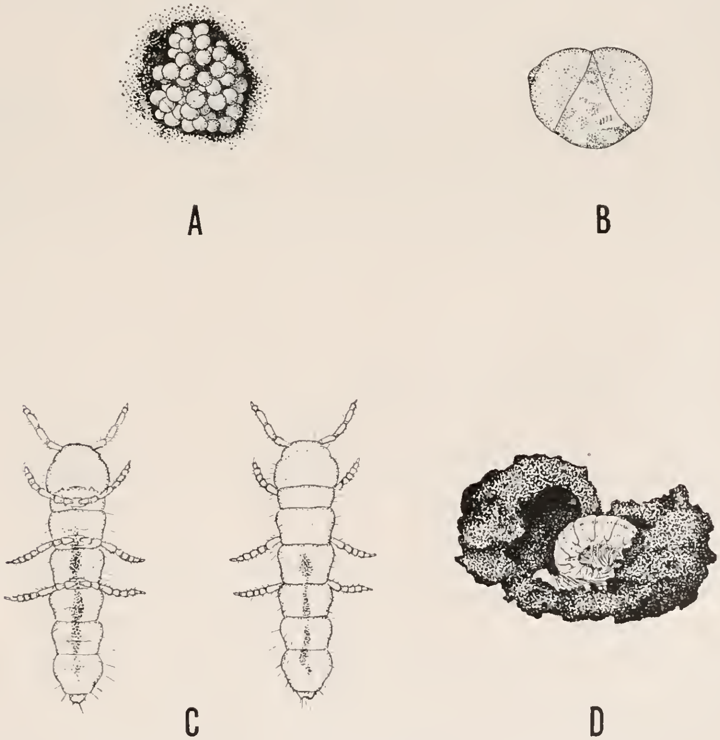

FIGURE 2. A. Egg-mass. Approx. × 2½. B. "Split-stage" egg. Approx. × 17. C. First instar larva in ventral and dorsal view. Approx. × 20. D. Opened cocoon of moulting larva. Approx. × 1¼.

loose cells of humus or in the closed ends of tunnel branches, a few inches below the surface. No firmly cemented cocoons of the type manufactured by moulting larvae appear to be constructed for these nests.

The eggs were approximately round, averaged 0.7 mm. in diameter, and appeared pearly, with a smooth surface. They were coated with a sticky substance that caused them to clump together, as has been described in other millipedes (Miley, 1927; Causey, 1943). As the eggs advanced in age the outer envelope took on a light brownish tinge.

In a number of egg clusters investigated, development had proceeded to a point which we have called the "split-stage" (Fig. 2B). In this stage, in which two halves of the split shell have been sprung apart like hinged hemispheres, there is contained a sac-like structure through which the developing larva with its appendages, etc., can just be perceived. Such a stage has been described for the juloid millipede, Arctobolus (Loomis, 1933) but not for the more closely related Polydesmid, Orthomorpha (Causey, 1943).

Loomis relates that in Arctobolus the first stage after hatching consists of a grub-like creature without appendages contained in a delicate semi-transparent membrane. We believe, although it could not be demonstrated, that in Luminodesmus this stage is passed briefly while in the "split-stage." When true hatching occurs, typical six-legged first instar larvae appear. In a large nest of "split-stage" eggs, first instar larvae were observed hatching from the membranous structure within the halves of the shell.

That the duration of time in the egg is not less than two weeks was demonstrated, since first instar larvae hatched from July 31st to August 3rd from "split-stage" eggs that had been isolated in small glass jars on July 19th.

No luminosity whatever could be observed in large egg-masses either before or in the "split-stage." All observations on luminescence were made in a dark-room after 15–20 minutes of dark-adaptation by means of Polaroid Corp. type-XDA8FAP red lenses. Single first instar larvae were checked for luminescence and none could be observed. An opportunity to check larvae hatching in numbers from a clump of eggs showed that enough luminescence is present in first instar larvae for it to be just apparent to the scanning, dark-adapted eye when the larvae are crowded together in a mass. Under these circumstances there is just enough luminosity present to be picked up with rod-vision in the thoroughly dark-adapted eye.

Whether luminosity is present in the egg itself has not been definitely determined. It is certainly not evident even when the eggs are in large masses; it is possible, however, that the opacity of the envelope prevents passage of light to the exterior.

The first instar larva may be seen in ventral and dorsal view in Figure 2C. This stage has 7 post-cephalic segments and averages 1.7 mm. in length. The animals are very active on hatching and move about feeding on the humus. At hatching they are transparently pale white, but shortly after commencing feeding, the gut, filled with black humus, becomes very evident.

A number of first instar larvae were isolated in humus on July 19th. By August 10th numbers of these were observed to have constructed the typical cemented cocoons in which moulting of larvae occurs. A cocoon broken open with a late larva *in situ* is shown in Figure 2D. The cocoon closely resembled that described by Miley (1927) in Euryurus. Cocoons are ball-like, smooth on the inside and rough on the exterior, of a dark, smoothly homogenous consistency apparently the result of the working together of humus and earth with some secretion. These structures are entirely closed and some possess the chimney-like extensions described by Miley. It is not unusual to find more than one individual in a single cocoon, particularly in some of the early moulting stages. As other authors have described, the millipedes are extremely inactive when in the process of moulting in the cocoons. That there is a general slowing down in metabolism at this time is indicated by the marked decrease in luminosity. No animals have been observed to lose their luminosity at any time from larva to adult, but during moulting the intensity is greatly diminished.

On August 20th the same isolation jar was examined, and a number of second instar larvae had appeared and were actively feeding. The time duration in the first instar was therefore approximately 33 days. Second instar larvae had 6 pairs of appendages, 9 post-cephalic segments and averaged 2.9 mm. in length. Total luminosity of these animals had increased so much that the position of three animals in a Petri dish could clearly be observed after 10 minutes dark-adaptation.

TABLE I

Characteristics of developmental stages

	Pairs of legs		No. post-cephalic segments	Average length in mm.
	♂	♀		
1st stage	3	3	7	1.7
2nd stage	6	6	9	2.9
3rd stage	11	11	12	3.8
4th stage	16	17	15	6.5
5th stage	22	23	17	8.0
6th stage	26	27	18	13.4
7th stage	28	29	19	22.1
Adult	30	31	20	41.0

On September 9th these second stage larvae were observed to have constructed cocoons and on September 20th a number of third stage larvae had appeared. These possessed 11 pairs of legs, 12 post-cephalic segments and averaged 3.8 mm. in length. The time duration of the second instar was approximately 31 days.

Subsequent moults appear to proceed in much the same way, although as this is written laboratory-raised larvae have only reached the fourth larval stage. Fifth, sixth and seventh stage larvae were collected in the field in May and October of 1951.

The characteristics of the developmental stages are summarized in Table I.

Gonopod development in the males of *Luminodesmus sequoiae* resembles that described for other polydesmoid millipedes. In the third larval stage no sexual dimorphism can be observed in the appendages. In the fourth larval stage, in place of the anterior pair of legs of the 7th post-cephalic segment there appear two very small raised domes (Fig. 3A). In the fifth larval stage these have become slightly larger. In the sixth and seventh larval stages proximal and distal segments are plainly apparent, the former segment being sunk into a pit in the sternite (Fig. 3B). The fully developed gonopod appears in the adult (Fig. 3C).

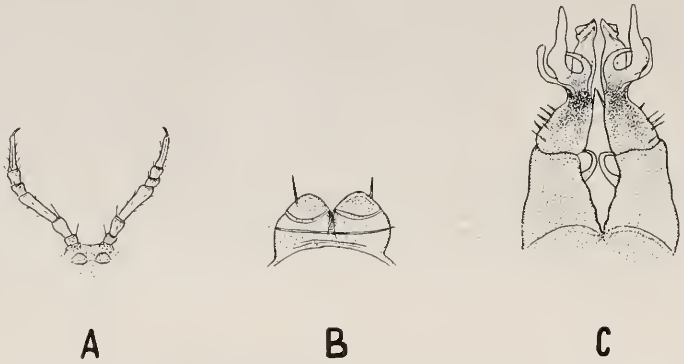

FIGURE 3. A. Appendages of the 7th post-cephalic segment in male fourth larval stage, showing gonopods appearing in position of eighth leg-pair. B. Developing gonopods of the seventh instar. C. Gonopods of adult male. All approx. × 13.

THE SOURCE AND NATURE OF LUMINESCENCE

While an extensive study of the luminescence of *Luminodesmus sequoiae* has yet to be made, some observations of general interest can be presented at this time.

The luminescence is continuous and appears to be under no voluntary control. During periods of general inactivity (*e.g.*, moulting) a decrease in intensity may occur. Momentary variations in light intensity in the entire animal may occur, but at the present time it cannot be said with certainty that these changes are correlated with changes in bodily activity. It has often been noted that luminosity may fade within segments or groups of segments under abnormal conditions (such as preceding death from local damage, poisons, CO_2).

Several observers have stated that they had an impression of a particulate light source when they examined the brilliant dorsal integument. Figure 4, an enlargement of five segments taken in their own light, strengthens the impression that there are in the integument scattered points of more intense luminosity. It is possible, however, that this effect may result from the breaking up of uniform light coming from deeper layers by the surface sculpture of the integument. This photograph, as well as Figure 1B, was taken in complete darkness with a Leica f-2 on Super-XX panchromatic film. The exposure time was one hour. Animals were inactivated by injection of 0.25 cc. *d*-tubocurarine chloride at a concentration of 3 mg./cc. The neuro-muscular blocking agent appeared to have no direct effect on luminescence.

Efforts to determine the exact source of the luminescence have been made as follows. Dissection and observations have determined that the fat bodies, viscera, tracheae, musculature and blood are not luminous. Frozen sections of adult animals have been made with CO_2. If these are rapidly thawed out under a jet of oxygen and observed in darkness, it can be seen that the source of the luminescence lies in the integument, since a cross-sectional "pipe" of light can be observed. The light from such sections fades out in a few minutes and is so pale and so diffuse as to make it impossible to resolve the source under the microscope. If the sclerotized integument of the animal is scraped with a scalpel on the outside so that flakes are removed, no trace of luminosity can be observed in these flakes. Further evidence that the source of the luminescence may lie in the deeper integumentary layers, perhaps the epidermis or endocuticle, is presented by the fact that the antennae and legs, containing non-luminous blood, muscle, etc., are brilliantly luminescent. Under the dissecting microscope in darkness, appendages appear to give a relatively uniform glow with the intensity appearing greater to the eye at the curving surfaces at the margins, where there is, of course, greater integumentary thickness.

Paraffin sections of adult animals and fourth stage larvae have been cut and stained with Mallory's triple stain with Orange G. It has so far been impossible to discern either in the endocuticle, which is traversed by numerous canals, or the epidermis, the granules which have so often been described in animal cells that are auto-luminescent. Fresh, unstained smears of integument or of appendages, or smears stained with methylene blue have given no indication under oil-immersion of the presence of such bodies or of bacteria which might be suspected of being the source of luminescence.

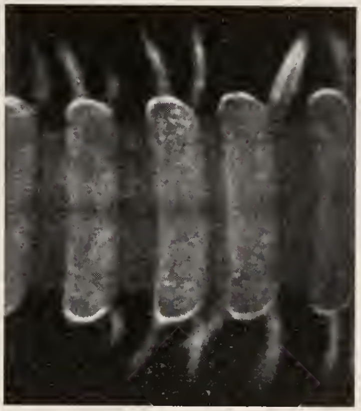

FIGURE 4. A dorsal view taken in animal's own light showing particulate appearance of light source and brilliance of lateral margins. Animal curarized. Approx. × 4.

Efforts to make extracts of Luminodesmus which would retain luminescence *in vitro* have so far been without success. Extracts prepared by grinding whole or eviscerated animals in water and filtering the extract into an evaporating dish glowed very briefly and went out. To date it has not been possible to re-activate the extract by adding various combinations of Photinus extract (including concentrated "luciferase" extracts made from freshly dried lanterns), magnesium ion and adenosine triphosphate (*cf.* McElroy and Harvey, 1951, for further references). Attempts to show classical "luciferin-luciferase" activity by combining heat-treated fractions with non-heat-treated ones (Harvey, 1940) have not been successful. However, it should be possible to study the effects of some factors on the emission of light in Luminodesmus in a semi-isolated system, since it has recently been found that single legs and antennae if placed in distilled water will retain full luminosity for at least four hours and overnight if refrigerated.

As has been stated (Loomis and Davenport, 1951), if the animal is irradiated with an ultra-violet light source, a brilliant chartreuse-green fluorescence appears. This fluorescence comes from the entire integument and often most brilliantly from areas which are not as intensely luminescent as others, such as the ventral surface. Blood, viscera and trachae are not fluorescent. That a yellow-green fluorescent compound is directly involved in the luminescence of fire-flies has been demonstrated (Strehler and McElroy, 1949). However, it should be borne in mind that many fluorescent compounds are known that are not concerned with the luminescent reaction.

That Luminodesmus, like most other luminous forms, must have oxygen in order to maintain full intensity of light production, was demonstrated by the use of nitrogen and carbon dioxide. Individual animals were placed in the bottom of a cotton-stoppered U-tube through which gases were drawn. The luminescence of animals in an atmosphere of pure nitrogen rapidly faded so that after five minutes only a dim glow was perceptible. This minimum light production was maintained as long as animals were observed (three hours). During this time the animals were quiescent, but apparently the effect on luminescence was specific, for the introduction of oxygen to the U-tube immediately restored full and brilliant light, while the animals returned to normal, unimpaired activity. Whether or not the dim light given off during prolonged exposure to nitrogen also requires small amounts of molecular oxygen remains to be investigated (*cf.* Harvey, 1940, p. 127).

Atmospheres of pure CO_2 were found to cause the rapid and complete disappearance of luminescence within two minutes and also to be extremely toxic. Animals exposed to the gas for 15 minutes not only failed to luminesce but were completely inactivated. Re-introduction of oxygen to the U-tube caused a partial return of light production almost immediately, but that this exposure to CO_2 was close to the lethal point was demonstrated by the fact that some animals failed to recover complete luminosity and activity, and ultimately died. Whether or not CO_2 has a specific effect on the luminescent system is yet to be demonstrated.

Animals were subjected to HCN in a collecting tube strong enough to kill Hymenoptera (Apis) almost instantaneously. The millipedes showed a marked decrease in activity at the end of an hour but no decrease in luminescence below the normal brilliant level. At the end of approximately three hours the animals were completely inactive (dead?) and the luminescence greatly reduced. Neither activity nor full luminescence ever returned when these animals were placed in an atmosphere of pure oxygen. These observations suggest that cyanide is relatively non-toxic to the luminescent system and does not affect it *in vivo* until the lethal point is reached. This is of interest in view of the demonstration of McElroy and Strehler (1949) that HCN not only fails to inhibit luminescence in luminous bacteria and in isolated biochemical systems but actually stimulates light production.

Finally it may be asked what the function of the luminescence is in Luminodesmus. It would appear that it cannot serve as a recognition factor, since all members of this millipede order are blind. As will be seen, Luminodesmus, like many other millipedes, gives off an offensive odor. Cook (1900) says "this absence of eyes also renders apparently meaningless the fact recorded by Bruner and Kenyon that the repugnatorial secretion of a Nebraska species" (*Fontaria luminosa* Kenyon) "is luminous . . . but when the nocturnal habits of the animals are considered, phosphorescence may be looked upon as affording a protection additional to that of the odor of the repugnatorial fluid."

This hypothesis that a "warning luminescence" has evolved in these millipedes can only be proved by the most carefully controlled field experiments involving nocturnal predators; at the present time it must be said that the role the light plays in the life of the animals is problematical.

Release of HCN

The release of HCN by millipedes has been noted by a number of authors in many species (Guldensteeden-Egeling, 1882; Verhoeff, 1928; etc.).

When collecting live Luminodesmus at the type locality and many times thereafter in handling experimental animals, observers have noted a powerful "cyanide jar" smell. It seemed advisable, therefore, to test for the presence of cyanide. Accordingly, the highly specific and delicate test developed by Fox (1934) was employed. Air is drawn through a system to be tested and then bubbled through an alkaline silver iodide suspension. If HCN is present, the KCN formed immediately dissolves the AgI, the cloudy bluish suspension disappears, and the resulting solution is perfectly clear.

If air was drawn over soil in which the millipedes had been living, a negative test resulted, but air drawn over a single animal which had been slightly agitated in a test-tube gave a strong positive reaction, indicating the release by the animal of HCN.

A biological test demonstrated the poisonous effects of the material released by the millipedes. Bees (Apis) were enclosed in test-tubes with millipedes and slightly agitated. Within three minutes the bees completely lost motor control and if enclosed thus for more than 15 minutes failed to recover. Control bees showed no diminution of angry activity if enclosed in similar stoppered tubes for half an hour.

No direct physiological connection between the phenomenon of luminescence and the release of HCN in Luminodesmus has been demonstrated. Kenyon (1893) and Cook (1900) discuss the possibility that in *F. luminosa* the repugnatorial glands are also the source of the "bead-like" luminosity. It would be of great interest to determine whether the glands of this animal, like those of Luminodesmus and other polydesmoids, release HCN and whether they are in fact involved in the luminescence, particularly in view of the demonstration of McElroy and Strehler, cited above, that cyanide stimulates the light-emitting reaction in other organisms.

Summary

1. The characteristics, distribution and habitat of the millipede, *Luminodesmus sequoiae* Loomis and Davenport are discussed. The life-history and the development of the male gonopods are described.

2. Luminescence is continuous, under no voluntary control, and first appears on hatching; its source apparently lies in the deeper layers of the integument.

3. Extracts of Luminodesmus glow briefly and go out. Experiments to elicit a light flash from such extracts with combinations of Photinus lanterns, magnesium

ion and adenosine triphosphate, and to demonstrate classical "luciferin-luciferase" activity have been without success. The integument of Luminodesmus gives a brilliant yellow-green fluorescence in ultra-violet light.

4. Nitrogen atmospheres reversibly extinguish luminescence, carbon dioxide irreversibly so, while cyanide has little effect on luminescence until the lethal point is reached. Chemical and biological tests have indicated that Luminodesmus, like other millipedes, releases HCN.

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THE RELATION BETWEEN OLFACTORY RESPONSE AND RECEPTOR POPULATION IN THE BLOWFLY

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In the course of an investigation to determine rejection thresholds of the blowfly, *Phormia regina* Meigen, for the vapors of homologous alcohols it became desirable to ascertain whether or not the response elicited depended entirely upon stimulation of the olfactory sense, as commonly believed, or represented instead a less specific action by way of a common chemical sense. In order to check this point it was necessary to measure accurately the threshold concentration for some test substance in the vapor phase and then to follow quantitatively changes in threshold value as different areas suspected of bearing olfactory receptors were extirpated. Execution of this plan showed not only that rejection was mediated almost entirely through the olfactory sense, but it yielded in addition some unexpected information relative to the comparative sensitivity of the different olfactory receptor fields and the importance of bilateral versus unilateral stimulation. This last is of particular interest if one is to give due consideration to the possibility of summing and to the relative contributions of the peripheral and central components of the nervous system to those aspects of behavior which depend initially upon stimulation of the sensory system.

Methods and Results

By analogy with other insects, the principal areas to be suspected of bearing olfactory receptors are the antennae, the labial palpi, and the labellum. Frings (1941) had shown by conditioning experiments that the olfactory sense of a related species, *Cynomyia cadaverina* Desvoidy, is resident in the antennae and labellum. Experiments by Dethier and Chadwick (1947) in another connection had shown that the response of *Phormia* to the odors of many organic compounds is abolished when the antennae and labellum are removed. The present investigation was designed to establish these results on a quantitative basis and to assess the contribution of the several sensory areas to the rejection response. Pentanol was selected as a test compound because of the decisive manner in which flies responded to it and the exactness with which a threshold value could be measured. The technique of threshold measurement has been described in detail in an earlier communication (Dethier and Yost, 1952).

For the present study, measurements of response were made with normal animals and animals from which head appendages had been removed by cutting, singly or in various combinations. Instead of varying the concentrations and determining what strength of stimulus was required to elicit 50% response (median rejection threshold) under each experimental condition, the simpler procedure was adopted of selecting two concentrations and determining the change in the per cent of the

TABLE I

Response of normal and operated Phormia to the vapor of pentanol

Receptor areas remaining	Mean % rejection (1)	Mean % rejection (2)	t value	Probability	Significance of difference
Tested at 5.0×10^{-6} *M*					
(1) All (normal) (2) Blank (pure air: no stimulus)	93.3	13.0	23.0	<0.01	+
(1) All except 1 ant. (2) Blank	69.3	11.8	11.3	<0.01	+
(1) Labellum and palpi (2) Blank	47.2	13.8	5.2	<0.01	+
(1) Palpi (2) Blank	34.5	12.7	3.5	<0.01	+
(1) None (2) Blank	15.7	3.0	2.8	0.025	slight
(1) All (normal) (2) All except 1 ant.	93.3	69.3	4.2	<0.01	+
(1) All except 1 ant. (2) Labellum and palpi	69.3	47.2	2.8	0.014	slight
(1) Labellum and palpi (2) None	47.2	15.7	4.4	<0.01	+
(1) Labellum and palpi (2) Palpi	47.2	34.5	1.6	0.15	–
(1) Palpi (2) None	34.5	15.7	2.6	0.02	slight
Tested at 1.6×10^{-6} *M*					
(1) All (normal) (2) Blank	32.3	6.6	4.9	<0.01	+
(1) Labellum and palpi (2) Blank	11.3	11.2	0.012	>0.9	–
(1) None (2) Blank	10.9	10.0	0.21	0.84	–
(1) All (normal) (2) Labellum and palpi	32.3	11.3	2.8	0.017	slight
(1) Labellum and palpi (2) None	11.3	10.9	0.052	>0.9	–

population responding as the various appendages were removed. Since the relation between the per cent of any population responding and the concentration to which it responds is known (the distribution is normal with reference to the logarithm of concentration), this method of approach is justified. The two concentrations selected were 5.0×10^{-6} *M* pentanol, to which approximately 90% of the population normally responds, and 1.6×10^{-6} *M*, to which approximately 25% normally responds. The median is 2.2×10^{-6} *M*.

The results, together with tests of significance, are listed in Table I.

Discussion

Three points for consideration emerge from an inspection of the foregoing data. These relate to the location of receptors, the differential sensitivity of receptor fields, and the difference between unilateral and bilateral thresholds.

When the higher concentration of pentanol is employed as a test stimulus, response in the form of rejection by the animal is abolished only when all of the head appendages (*i.e.*, antennae, palpi, and labellum) are destroyed. On statistical grounds there is still a slight possibility of a retention of some residual sensitivity even then to this concentration (see Table I, "Tested at 5.0×10^{-6} *M*, No Receptors Remaining"), but the degree of response is very low. The reduction of response is most marked following removal of the antennae. No difference in response could be detected between animals possessing labella and palpi only and those possessing palpi only. Therefore, receptors sensitive to the lower concentrations of pentanol are presumed to be present on the antennae and palpi.

This conclusion is in conflict with those previously reported by Dethier and Chadwick (1947) and Frings (1941). Part of the discrepancy may be resolved by giving attention to the problem of stimulus concentration. When a weak stimulus is employed for testing, response is completely abolished following removal of antennae alone (Table I, "Tested at 1.6×10^{-6} *M*, Labellum and Palpi Remaining"). In the earlier experiments with *Phormia*, where the responses of normal versus antennectomized-labellectomized flies were compared, it had been observed that the latter failed to respond to the odors of solutions employed in stimulating tarsal chemoreceptors. The test odors, although not quantitatively controlled at that time, were of low concentration, and a repetition of the experiments has subsequently shown that antennectomy alone is sufficient to prevent the olfactory response of flies under those conditions of testing.

The data for *Phormia* and those obtained by Frings with *Cynomyia cadaverina* could be reconciled by an explanation which would take advantage of the species difference, but this is admittedly unsatisfactory. On the other hand, it is conceivable that the differential sensitivity of the various appendages in the two species is not identical. If receptors sensitive to stimulation by compounds in the vapor phase were considered to be present on all three sets of appendages, the comparative sensitivity in the two species might be antennae > labellum > palpi in *Cynomyia* and antennae > palpi > labellum in *Phormia*. If the stimulus were of moderate to low concentration, only the antennae and labellum would be involved in the first species and the antennae and palpi in the second. Knowledge of such relationships would emerge only after a careful study of the entire concentration range. The fact remains, nonetheless, that *Phormia* is repelled by low concentrations of gaseous substances only when both antennae are intact and by high concentrations only when the antennae or palpi are intact. Therefore repellents in the vapor state may be presumed to be acting on olfactory receptors.

Differences between responses at high and low concentrations of pentanol show quite forcefully that all receptor fields are not equally sensitive and that the field on the mouthparts is much less so than that on the antennae. Additional confirmation for this last conclusion is seen in the degree to which response is reduced following removal of different appendages. The greatest reduction follows bilateral antennectomy. This confirms what has been known for some time, that the antennae of insects are generally sensitive to lower concentrations of odors than other receptor fields. Whether the difference is to be ascribed to a difference in the number of receptors involved, in their intrinsic thresholds, or in their central nervous connections cannot be known at this time.

The possibility of a dependence of total response upon activation of a threshold number of receptors has not been carefully examined in olfaction. The phenomenon has been investigated to some degree for the humidity sense. The suggestion that a threshold number of receptors is required for a response to humidity changes was first demonstrated by Pielou (1940) on evidence gathered with the mealworm, *Tenebrio molitor*. Detailed confirmation was provided by the experiments of Roth and Willis (1951a) which showed that the per cent of response of a population of two species of *Tribolium* was closely correlated with the number of sensilla basiconica remaining on each individual after surgical operation. As the number was progressively decreased the per cent response to humidity differences correspondingly decreased.

The present data on olfactory response to high concentrations of pentanol permit an extension of the analysis of the relation between receptor number and threshold of response. The existence of such a relation is indicated by the significant difference in response between those flies possessing both antennae and those with a single antenna. Observation of individual flies, plus the fact that the response of flies with a single antenna could be made to match the response of normal flies by adjustment of the stimulating concentration, indicated that results could not be attributed to interference with locomotory behavior.

If one makes the assumption that x number of receptors must be activated to insure a response, then at the median threshold value 50% of the flies have x or more receptors acting. The probability at this concentration that a fly (x or more receptors) will be in the half of the population which is responding is 50%. This probability can be increased either by increasing the concentration or by increasing the number of available receptors (n). If n is doubled, the probability of a response increases. This increase can be calculated from the expression $1\text{-}q^2$ where q is equivalent to the per cent not acting at the median concentration. Accordingly, the concentration which elicits a response from 50% of the flies possessing a single antenna should elicit a 75% response from the flies possessing both antennae if there is no interaction between the two antennae.

Although the concentration chosen to test this hypothesis was the 90% level rather than the 50% level, use of the expression $1\text{-}q^2$ still yields the desired results. Application of the test to the data shows that the decrease in threshold with two antennae as opposed to one can be accounted for satisfactorily on a probability basis.

Since the probability of a response can be increased (reflected as a decrease in threshold concentration) either by increasing the receptor number or by increasing the concentration and holding the receptor number constant, it may indeed be concluded that some relation exists between concentration and number of receptors activated. Furthermore, it can be said that as the concentration is increased a larger number of receptors is activated. Beyond this one cannot go on the basis of these data.

TABLE II

Effects of unilateral and bilateral operations in the responses of beetles given a choice between 0% and 100% R.H. at 27° C.
(Data from Roth and Willis, 1951b)

Species	Receptor areas remaining on antenna	% response
Rhyzopertha dominica (thin-walled sensilla present on segments 8 to 10)	Segments 1–10 on both (normal)	76 ± 2.7
	Segments 1–10 on one Segments 1–9 on other	69 ± 3.0
	Segments 1–9 on both	56 ± 3.4
	Segments 1–10 on one Segments 1–7 on other	61 ± 2.9
	Segments 1–7 on both	−0.1 ± 7.0
Latheticus oryzae (thin-walled sensilla present on segments 7 to 11)	Segments 1–11 on both (normal)	87 ± 3.1
	Segments 1–11 on one Segments 1–10 on other	73 ± 3.2
	Segments 1–10 on both	46 ± 2.7
Tribolium castaneum (thin-walled sensilla present on segments 9 to 11)	Segments 1–11 on both (normal)	80 ± 1.0
	Segments 1–9 on both	75 ± 2.9
	Segments 1–9 on one Segments 1–8 on other	78 ± 3.6
	Segments 1–8 on both	31 ± 5.4
Tribolium confusum (thin-walled sensilla present on segments 7 to 11)	Segments 1–11 on both (normal)	86 ± 1.7
	Segments 1–11 on one 0 segments on other	76 ± 3.0
	0 antennae	−0.2 ± 2.2
	Segments 1–7 on both	23 ± 1.6
	Segments 1–7 on one Segments 1–6 on other	12 ± 3.9
	Segments 1–6 on both	5 ± 4.1
	Segments 1–11 on one Segments 1–6 on other	75 ± 2.1
	Segments 1–6 on both	5 ± 4.1

Rise of threshold with unilateral extirpation has not been emphasized before, but it is implicit in many recorded data. It is seen, for example, in the humidity responses of many beetles. Several selected data of Roth and Willis (1951b) show it clearly (Table II). Here the response is always (with the one exception in the case of *Tribolium castaneum*) reduced when one antenna or portion thereof is removed. The response is further diminished when corresponding areas on the remaining antenna are removed. In none of these cases, nor in the olfactory data presented, does the experimental value of response to bilateral stimulation exceed that for unilateral stimulation by a value greater than that which can be accounted for by statistical bias. Consequently, it can be stated that there is here no evidence for contralateral neural summation (*cf.* Dethier, 1950). However, when only one or two concentrations rather than a complete frequency distribution have been investigated, it is not possible to analyze the situation fully. Nor can one apply a rigorous test of significance to the difference between the experimental value for bilateral stimulation and the calculated value and consequently settle the question of summation with any great degree of certainty. This follows in part because the Chi square test is inaccurate when small numbers are used and only one degree of freedom is involved.

Since decrease of unilateral threshold over bilateral threshold has not been recorded as occurring commonly among animals (the difference between monaural and binaural thresholds, and monocular and binocular thresholds in man being two of the better known examples), the phenomenon is worthy of closer scrutiny. Additional experiments with the tarsal chemoreceptors, which are more amenable to analysis, are in progress to settle this point. At the moment it would appear that the results reported here do not indicate the existence of true neural summation but instead what has been termed supplementation by Smith and Licklider (1949). Nonetheless, insofar as interaction exists between bilaterally placed receptor fields, it remains from the point of view of the integrated organism a behavioral summation of no little importance.

SUMMARY

1. Measurements have been made of thresholds of rejection of two concentrations of pentanol vapor by normal blowflies and those from which various head appendages have been removed. The concentrations selected were 5.0×10^{-6} *M* pentanol, to which 90% of the population normally responds, and 1.6×10^{-6} *M*, to which approximately 25% of the population normally responds.

2. The results show that the response elicited depends upon stimulation of specialized receptors present on the antennae and palpi and hence cannot be attributed to the action of a common chemical sense. Repellents in the gas phase undoubtedly act as olfactory stimuli. Different receptor fields function at different levels of sensitivity. The antennae are the most sensitive and the various mouthparts less so. There is also a relation between threshold and the number of receptors functioning. Insects possessing both antennae respond to lower concentrations of odor than those bearing only one. This indicates that the two sides of the body are acting in concert. Whether it represents true neural contralateral summation or is based upon statistical bias cannot yet be decided with certainty although the data strongly suggest the latter. In either case, from the point of view of coordinated behavior it illustrates the manner in which bilaterally placed receptor fields may operate as a unit.

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THE DIGESTION AND ABSORPTION OF FAT IN LAMELLIBRANCHS[1]

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The problem of digestion in lamellibranch mollusks has been a subject of interest to biologists for many years. It seems well established that the digestion of carbohydrates takes place largely extra-cellularly in the lumen of the stomach through the action of enzymes liberated through the dissolution of the crystalline style. The place and mode of digestion and absorption of proteins and fats have occasioned disagreement. One concept supported especially by a series of papers by Yonge (1923, 1926a, 1926b, 1930, 1946) holds that the digestion of fats and proteins is exclusively or largely intra-cellular, occurring in phagocytes of the blood and in cells of the digestive diverticula. Another group of investigators has questioned the correctness of this view and has presented some evidence to indicate that the digestion of fats and proteins occurs extra-cellularly through the action of enzymes of uncertain source (Swano, 1929; Nelson, 1933; Mansour, 1945, 1946; Mansour-Bek, 1945, 1946).

The present author's concern with the structure and primitive functions of blood and hemolymph gives the problem of digestion in invertebrates a special interest. The thesis of intra-cellular digestion in leucocytes was appealing and tentatively accepted (George, 1941). That digestion by phagocytes of the blood was so extensive as to be a significant feature of the digestive process did not appear soundly enough established, however, to justify its acceptance as a basis for generalization. The present investigation, which is limited to the digestion and absorption of fats, was undertaken during July–August, 1950, to determine the extent of participation of leucocytes in the digestive process of some lamellibranchs.

MATERIAL AND METHODS

Several species of lamellibranchs were used but principally the common oyster, *Crassostrea virginica*, and the ribbed mussel, *Modiolus demissus*, both abundantly available in Beaufort Harbor. Stained neutral fats (olive oil and peanut oil) were fed as emulsions stained with Sudan black B or with Sudan III (Scarlet red). Scarlet red,[2] which stains both neutral fats and fatty acids pink, was chosen for most experiments because of its advantage in subsequent counter-staining with Nile blue sulfate. Emulsions of the oil, previously stained to saturation, were prepared as follows: 60 cc.

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[2] The scarlet red used in this investigation was given to the author a few years ago by the late Simon Henry Gage and is from the lot used by him and Fish in their investigations.

of the stained oil, 15 grams of powdered acacia and 30 cc. of distilled water were put in a Waring Blendor and thoroughly mixed. The emulsion was then thinned somewhat by the gradual mixing in the Blendor of 20 cc. additional distilled water. This produced a satisfactory stock emulsion with particles ranging in size from less than 1 μ to perhaps 10 μ. Oil immersion examination of a diluted drop of the stock emulsion shows the larger droplets to have a clear pink color; a slight but definite pink is recognizable in medium sized droplets; no definite color is detectable in individual droplets of the smallest size. In some cases the emulsion was inserted with a pipette through the mouth into the stomachs of animals after the removal of one valve. Sometimes animals were fed in salt water aquaria. A few drops of the emulsion poured into the water, kept turbulent by bubbling air through it from an air compressor, is quickly distributed throughout an aquarium. As some of the oil slowly accumulates at the surface, this can be skimmed off and the concentration restored by the addition of fresh emulsion.

Fat droplets withdrawn with a pipette from the stomachs at definite times after feeding, or droplets taken from other experimental material can be identified by color when examined with the microscope. By counter-staining with a solution of Nile blue sulfate allowed to run under from the edge of the cover glass, one can determine if any hydrolysis of neutral fat has occurred. Droplets that have undergone reduction to fatty acid are changed from pink to a clear blue as the Nile blue sulfate reaches them, whereas droplets of neutral fat become deeper pink (an orange pink). The same microscopic picture is obtained after exposure of emulsion to a solution of commercial lipase (steapsin).[3] No droplets of the untreated stock solution turn blue when counter-stained with Nile blue sulfate; on the contrary they all turn a deeper pink. As a counter-stain, concentrations of Nile blue sulfate ranging from 1 : 1000 to 1 : 300,000 were used and proved effective. A solution of 1 : 10,000 was settled on as most satisfactory.

Tests for lipase in styles and various other structures were made by mixing small quantities of stock emulsion with a mince of the structures in 2–3 drops of sea water or tap water on a hollow ground slide and subsequently counter-staining a sample with Nile blue sulfate. Generally no bactericidal agent was used; similar results were obtained when a drop of 0.5% phenol solution was added to 2 drops of water of the mince.

Material for sections was fixed in 10% formalin in sea water.

Results

Oysters and mussels kept for as long as five days in turbulent water with a suspension of olive oil emulsion stained with Sudan black showed no deposition of stained fat in any of the storage tissues although the emulsion was freely taken into the gut. Similar experiments with emulsions of peanut oil stained with scarlet red likewise gave no evidence for storage of stained fat such as Gage and Fish (1924) found in mammals and birds.

Although these experiments gave no evidence for storage of experimental fat in the tissues, the evidence for digestion within the stomach seems clear. In emulsion withdrawn and tested after having remained in the stomach for only a short time, some droplets became a pale blue after Nile blue counter-staining, indicating the beginning of fat digestion, but many droplets retained the pink color of neutral fat. In emulsion that had remained in the stomach for several hours, the number of blue droplets after Nile blue sulfate was much greater and the depth of blue was deeper, most drops showing an intense blue color. In emulsion withdrawn and tested after a still longer time in the stomach, some of the larger droplets had a shrunken appearance, due, perhaps, to the diffusion of glycerol from the drops; and after 30 hours exposure to stomach juice some droplets contained needle-like crystals, probably fatty acid crystals.

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[3] Lipase (steapsin) from Nutritional Biochemicals Corporation was used.

My findings with regard to the digestion of fat in the stomach seem to be in conflict in some essential matters with the conclusions of Yonge (1926b), who fed olive oil stained red with Nile blue sulphate to oysters. On p. 351 he says, "In the lumen of the stomach there were immense numbers of phagocytes, most of them with ingested oil. In certain cases they collected in great numbers round large droplets of oil, which had turned blue under the influence of their enzymes. *All oil droplets lying free in the stomach and not surrounded by phagocytes retained the red color*—evidence of the absence of lipase in the stomach." The presence of phagocytes and their ingestion and digestion of oil I can confirm; but the absence of digestion of oil other than under the influence of phagocytes is not in accordance with my observations. It is conceivable, of course, but not likely that *Ostrea edulis* and *Crassostrea virginica* differ markedly in their physiology of fat digestion and absorption, but it seems more probable that the oil observed by Yonge had been inadequately exposed to the action of stomach juices.

If the disintegration of leucocytes liberates appreciable amounts of lipase or if they secrete it, it seems reasonable to assume that some should be present in the plasma. To test for lipase in plasma, fat emulsion diluted with sea water was injected into the hearts of several oysters and mussels and one clam. In all cases, tests for fat digestion after two to ten hours were positive so far as digestion within blood cells was concerned and negative for extra-cellular digestion in plasma. Also, hanging drop cultures of fat emulsion mixed with blood were made. Examination after one hour showed ingestion and digestion of pink fat droplets within leucocytes. No evidence for fat digestion could be found in the droplets that remained in the plasma. Further tests for lipase in plasma were made during August, 1951, by placing drops of plasma from fresh water mussels, salt water mussels and oysters upon smears of margarine, unstained peanut oil, and Sudan III-stained cotton seed oil. This was to check the technique and results of Yount (1950), who reported the digestion of neutral fat by the plasma of oysters, mussels and clams. My several experiments were negative, no droplet ever turning blue after Nile blue except in the case of one batch of oil stained with Scarlet red the previous summer and carried over. In smears from that lot of oil, some blue droplets appeared after counter-staining with Nile blue. It turned out, however, that blue droplets appeared outside the areas to which plasma was applied as well as inside them and, indeed, in smears to which nothing was applied except Nile blue. Evidently that particular batch of oil was rancid, and it seems possible that Yount's positive results were due to rancid fat in his margarine. These observations seem to establish two points: 1) the leucocytes produce their own lipase, and 2) there is no evidence that they liberate any into the plasma (or, presumably, into stomach juice).

To determine the source of the lipase of the stomach juice, a drop of fat emulsion was added to minced crystalline style in two or three drops of sea water. Without exception these tests were positive for lipase activity. The specimens tested consisted of 12 oysters, 15 mussels, one clam (*Venus mercenaria*), one *Atrina livida,* and one razor clam (*Ensis directus*).

Two typical case reports from my notes will illustrate the progress of digestion. *Mussel MA*—style was removed, washed, and minced in 2 drops of sea water; a drop of emulsion was added at 9:15 A.M. At 11 A.M. tests with Nile blue sulfate showed 10–15% of oil droplets to be blue; at 12 M. about 25%; at 2:30 P.M. almost 100%. *Oyster 27-B*—style minced in sea water at 11:45 A.M. At 12:30 P.M. 75% of the droplets are blue after Nile blue; at 4:45 P.M. 100% are blue. There is variation among individual oysters and mussels in the speed of the digestive action, or in the percentile response of fat droplets. These experiments indicate that in all these species of lamellibranchs the style is an important source of lipase.

To determine if active lipase was present in the digestive diverticula, fat emulsion was mixed with mince of a chunk of the glandular mass in two or three drops of sea water. Tests with Nile blue sulfate were made after varying times with the following results: all 9 oysters tested and the single *Atrina rigida* were negative for fat digestion; all 10 mussels tested and the single clam and the razor clam were positive. In the case of one mussel, the lining membrane of the stomach and the glandular mass were dissected apart, minced and tested separately. The glandular mass was positive and the lining membrane was negative for lipase. Although the tests for fat digestion by mince of the digestive diverticula were clearly positive in some species, they were less strongly so than was the case with the styles. The conclusion seems justified, then, that the principal source of lipase for extra-cellular digestion in the stomachs of the lamellibranchs studied is in the crystalline styles. Apparently this source may be supplemented in some species with lipase secreted by glandular tissue of the digestive diverticula.

Observations were made to determine if fat is transported from the lumen of the gut into the tissues by free phagocytes as supported by Yonge (1926b) and Takatsuki (1934). It seems reasonable to expect to find some if they are to be looked upon as significant agents in digestion and absorption. Oysters that had been kept in a suspension of stained fat emulsion in sea water were sacrificed at 8, 18, 21, 22, 25, 29, and 30 hours. Considerable fat was in the guts of these animals. Careful examination revealed no leucocytes with ingested experimental fat when blood was withdrawn from the hearts. In most specimens a few cells were found with apparent oil droplets, but no pink color was identifiable. These droplets stain blue with Nile blue, as do fatty acids (and intrinsic granules of oyster blood cells). In the connective tissue surrounding the gut epithelium, free cells with globules of a probably fatty nature were present but in no case could it be determined that they contained experimental fat. On the basis of the evidence one could say only that the blood cells may possibly transport a small amount of fat into the blood and tissues but the amount of transport is not enough to be significant in total nutrition.

Is the neutral fat used in these experiments absorbed at all and if so, how? To get an answer to these questions, sections of stomachs of experimental and control oysters were made by the freezing technique. Examinations of unstained sections from animals fed fat emulsion showed abundant small granules or droplets in the ciliated epithelium of the stomach and in the ciliated epithelium of the ducts of the digestive diverticula. Under high powers of the microscope these droplets individually show no color; but under low power they give a diffuse pinkness to the epithelial layer. There is little doubt that they are from the stained fat fed the animals. A secondary staining of the section with an alcoholic solution of Sudan III colors these droplets a bright orange. Counter-staining with Nile blue sulphate colors them a deep blue. The conclusion seems justified that they are droplets of the stock emulsion reduced to fatty acid and in process of being absorbed through the epithelium. After secondary staining with Nile blue or Sudan III it is clearly seen that the droplets are most abundant in the apical one-third to one-half of the columnar epithelial cells and least abundant in the middle third, a distribution accounted for in part, at least, by the fact that the nuclei occupy a large part of the middle third of the epithelial layer. Cells of some areas of the mucosa contain more droplets than other areas.

Frozen sections of control animals, neither fed nor starved, showed some sudanophil droplets in the epithelium but no such great accumulation as in the case of the animals fed oil.

What appear to be blood cells are to be found sometimes in the epithelium of both experimental and control animals. They are not present in sufficient numbers, however, as to justify corroboration of the conclusions of Yonge (1926b) and Takatsuki (1934) that wandering phagocytes are important agents of absorption in the gut. On the contrary, the facts from these experiments seem to lead to the conclusion that neutral fat fed to these lamellibranchs is reduced to fatty acid in the lumen of the gut and absorbed as independent particles through the ciliated epithelium of the gut and ducts of the digestive diverticula, and that little if any is carried through by leucocytes.

The epithelium of the tubular alveoli of the digestive diverticula shows two types of cells which tend to be associated in groups: 1) cells with coarse to fine granules of greenish brown color, and 2) smaller cells with smaller colorless granules of approximately uniform size, which Yonge (1926a) considers to be immature cells. Neither of the types of granules in these cells stains with alcoholic Sudan III. After Nile blue sulfate the greenish-brown granules become deep green; the colorless granules found in the smaller cells do not stain. No fat droplets could be discovered within or between the epithelial cells of the tubular alveoli as were found in the ciliated epithelium of their ducts and of the stomach. Hence, the results of feeding emulsified oil to oysters provide no evidence for the ingestion or absorption of fat particles by the epithelium of the digestive tubules.

What evidence do these experiments reveal concerning the storage of fat in the connective tissue around the gut? The connective tissue of lamellibranch mollusks contains large polygonal vacuolated cells with centrally located nuclei having a zone of perinuclear cytoplasm with strands radiating outward to a zone of peripheral cytoplasm. The vacuolar spaces within these cells contain few or many droplets of various sizes which in unstained sections are colorless. Sudan III in 70% alcohol colors them bright orange; Nile blue sulfate colors them blue. They are dissolved by higher alcohols and xylene. They probably contain stored fatty acid, but in unstained frozen sections of experimental animals no pink color could be detected in these droplets to identify them as having been derived from the stained emulsion fed the oysters. In addition to the fixed vacuolated cells of the connective tissue, large wandering cells are present. They contain drop-like inclusions of different sizes ranging in color from a clear brown to a pale color that might be mistaken for pink. They contain in addition small, relatively uniform granules resembling the intrinsic granules of cells of the blood. Droplets and granules both stain a blue green with Nile blue sulfate but they do not stain with alcoholic Sudan III. They are not dissolved by alcohols and xylene, and they are present in animals not fed fat emulsions as well as in those that are. It is suggested that these inclusions are not neutral fat or fatty acid but probably chromolipoids, perhaps mixed with other substances.

Discussion

Evidence from this investigation for the extra-cellular digestion of fats in the stomachs of oysters and mussels seems unequivocal and conclusive. Previous evidence has been contradictory. Claude Bernard (1855) reported that the stomach juice of oysters digests starches and fats. Albrecht (1921) found that extracts of the intestinal tracts of the clam, *Tivella stultorum,* have lipolytic action. More recently Mansour-Bek (1945) reported lipolytic activity by digestive juice pipetted from the stomachs of *Tridacna elongata* and *Pinctata vulgaris.* On the other hand, Yonge (1926a, 1926b, 1930, 1937, 1946) supports the thesis that in lamellibranchs digestion of proteins and fats is exclusively intra-cellular, the only extra-cellular enzymes normally present being those set free when the head of the crystalline style is dissolved in the stomach. He finds these enzymes to be exclusively amylolytic (Yonge, 1923, 1926b). Any proteolytic or lipolytic action found in filtered stomach juice is attributed to cytolysis of phagocytes. In this view he is supported by Graham (1931). (Other pertinent references are to be found in the papers cited above.)

Correlated with the differences of opinion in regard to intra-cellular and extra-cellular digestion in lamellibranchs is a corresponding lack of harmony concerning the enzymatic activity of the crystalline style and of the digestive diverticulum. It has become well established that the dissolution of the style liberates amylolytic enzymes. With regard to other enzymes the observations of workers are at variance. Yonge in a number of papers contends that the style consists largely of amylolytic enzymes and that no other enzymes are present in it. Chestnut (1949) with the methods he used found amylolytic but no demonstrable proteolytic or lypolytic activity in extracts of the crystalline style. Some other investigators get results not entirely in accord with Yonge's. Sawano (1929) reported the presence of lipase as well as amylase and protease in extracts of the crystalline style. Fox and Marks (1936) also attributed lipase as well as carbohydrases to extracts of the style of *Mytilus.* In discussing the digestion of animal forms by the oyster, Nelson (1933), without specifying the nature or the source of the active substances, states that "It is evident therefore that some substance or substances present in the stomach of an oyster with a well formed style can penetrate the chitin of crustacea and the cuticle of nematodes resulting in death and disintegration of the animals." Mansour (1946) and Mansour-Bek (1946) seem to consider that globules pinched off or shed from cells of the digestive gland are the source of extra-cellular lipolytic and proteolytic enzymes.

The experiments and observations reported in a previous part of this paper have an important bearing on certain features of these arguments. Sensitive tests show that hydrolysis of neutral fats does occur extra-cellularly in the cavity of stomachs of oysters and mussels. These tests also give a positive reaction for the presence of lipase in aqueous extracts of the crystalline style, which appears to be the principal source of the enzymes that mediate the hydrolysis of fat in the stomach. It is possible that lipolytic enzymes from the digestive diverticulum supplement those from the style, since minced diverticulum in water gives a positive reaction for the presence of lipase in individuals of some species, not all. One should not overlook the possibility, however, that normally this demonstrated lipase from the digestive gland might never become an extra-cellular enzyme but function intra-cellularly.

It is still an open question whether the tubules of the digestive gland are secretory or whether they exclusively absorb and digest phagocytically. A detailed exploration of the functions of the digestive gland was not a part of this investigation. However, some observations are significant. We have seen that the gland contains, possibly secretes, lipase in some species, but none was demonstrated in the oysters of these experiments. Chestnut (1949), working with the same species of oyster, found that extracts of the digestive diverticula have lipolytic activity as well as amylolytic and proteolytic activity. This difference in results suggests the possibility of seasonal variation in the presence of active enzymes or perhaps a difference in the effectiveness of different techniques in extracting enzymes from the cells. Phagocytic action is suggested by the fact that cells of the tubules contain what appear to be bits of foreign debris and greenish-brown granules of an organic nature that may have been ingested but probably were manufactured. These experiments produced no positive evidence and little circumstantial evidence that cells of the tubules ingest fat emulsion. It could be that the tubules of the digestive diverticula are specialized for the phagocytosis or absorption of some substances, not of others. Many authors have attributed an absorptive function to them. Saint-Hilaire (1893) found that the cells of the tubules of some lamellibranchs and other mollusks are not ferment cells but absorptive cells. Enriques (1902) and Gutheil (1912) consider that they absorb but that their main function is excretory. List (1902) found that in *Mytilus* particles of India ink were taken from water containing it by cells of the digestive diverticula. Vonk (1924) got similar results with oysters. From microscopic examinations of glands during the later stages of digestion in *Ostrea* and *Modiolus,* Nelson (1925) was led to believe that in these forms even more food is digested in the digestive glands than in the stomach. Yonge (1926a), by feeding iron saccharate to various lamellibranchs, found that the substance was taken up by amoebocytes and by cells of the tubules of the digestive diverticula but by no other part of the gut. Coe and Fox (1944) state that in *Mytilus californianus* "Many of the smallest objects and particles are phagocytized by the cells lining the digestive diverticula."

As regards the processes of absorption of fat, the observations here reported indicate that it is largely absorbed as particulate matter through the ciliated epithelium of the gut and the ducts of the digestive diverticula. None was identified in the cells of the tubules themselves. All fat observed in the process of being absorbed gives the reaction for fatty acid. There is no evidence for or against absorption as soluble fat, although it seems reasonable to assume that the glycerol component is so absorbed. Although leucocytes free in the lumen of the gut may ingest fat droplets in quantity, the evidence does not indicate any considerable transport of fat by them into the tissue since no leucocytes with identifiable experimental fat could be found in the blood or tissues. Furthermore, in confirmation of the observations of others, in both oysters and mussels I have observed many leucocytes in the lumen of the gut with ingested diatoms; but I have never seen any in the blood or tissue spaces, nor, so far as I am aware, has any one else. However, Gutheil (1912) figures and describes blood cells with food balls passing from the gut into the connective tissues. Yonge (1926b) did report that cells with ingested fat moved back into the blood spaces and tissues in *O. edulis.* I cannot confirm it for *C. virginica.* Wandering cells with lipoidal inclusions characteristically present in the connective tissues may have misled observers. As pointed out elsewhere, these lipoidal inclusions in *C. virginica* differ from the experimental fat in certain respects: they stain blue-green instead of blue with Nile blue sulphate; they do not stain with alcoholic Sudan III, and they are insoluble in absolute alcohol and xylene.

In spite of the evidence for digestion and absorption of stained emulsified fat, why was no evidence obtained in regard to the deposition of stained fat in the tissues of oysters and mussels? Gage and Fish (1924) got clear-cut results with birds and mammals and were able to follow stained fat through two generations. Three possible explanations come to mind: 1) oysters and mussels may be unable to re-synthesize the fatty acids of olive oil and peanut oil into their own specific fat, since the fatty acids of these oils differ from the unsaturated fatty acids of plankton on which the lamellibranchs feed (Markley, 1947); 2) fat may be deposited in connective tissue cells in such small discrete units that the pink color of the droplets is not distinguishable under the microscope, and the total amount of fat in an area may not be massive enough to be detectable on gross examination; 3) at the time of these experiments the stained fat absorbed may have been rapidly metabolized rather than stored. At certain periods, during the summer or after the development of spawn or fattening in the fall, fat is apparently rapidly metabolized. Nelson (1951) reports that great quantities of fat and oil can be demonstrated in New Jersey oysters in late spring before the development of spawn, and in late summer and early fall prior to the "fattening" which follows summer spawning. He suggests that this may be preliminary to the rapid build-up of glycogen, in which the storage in the gland of substantial quantities of oil constitutes the first step. This point deserves further investigation.

SUMMARY

Emulsions of olive oil and peanut oil stained with Sudan stains were fed to oysters and mussels and mixed with mince of crystalline styles and other structures. Samples of the experimental material were subsequently examined to determine if the fat was absorbed and how, and counterstained with Nile blue sulphate to determine if the neutral fat had been split to fatty acid and glycerine. The following observations and conclusions resulted:

1. Free droplets of neutral fat are hydrolyzed in the stomach.
2. Droplets of the stained fat in the form of fatty acid appeared in large numbers in the ciliated epithelium of the stomach and ducts of the digestive gland. None was found in the non-ciliated epithelium of the alveoli of the digestive gland.
3. Droplets of the emulsion were ingested by leucocytes in the lumen of the gut. Neutral fat in leucocytes was hydrolyzed. There was no certain evidence of the passage of any leucocytes with ingested fat back into the blood spaces or tissues.

4. Emulsion injected into the cavity of the heart or mixed in hanging drops of blood was ingested by leucocytes. Hydrolysis occurred within blood cells. No evidence was found for the hydrolysis of neutral fat by the plasma.

5. Tests of stained emulsion subjected to the influence of minced styles showed hydrolysis of neutral fat in all cases.

6. Emulsion subjected to the influence of minced digestive gland was positive for the hydrolysis of neutral fat in some species, but not in others.

7. No evidence for deposition of stained fat in the tissues was found. Possible explanations are suggested.

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THE EFFECT OF INGESTED RADIOPHOSPHORUS ON EGG PRODUCTION AND EMBRYO SURVIVAL IN THE WASP HABROBRACON [1, 2, 3]

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The present report is the first of a series concerned with investigations of the developmental and genetic effects of ingested radioactive isotopes. Although an appreciable amount of information is becoming available on the vertebrate histopathology of internal radioactive isotopes, apparently there have been few studies directed toward the cytogenetic consequences. Even in such organisms as insects, which produce large samples of offspring in a relatively short time, available data are limited to a few short papers on Drosophila (King, 1949; Bateman and Sinclair, 1950; Blumel, 1950).

The insect chosen for present investigations, an ectoparasite of Ephestia caterpillars known in genetic literature as *Habrobracon juglandis* (Ashmead), has the Hymenopteran advantage for induction experiments of parthenogenetically produced, genetically haploid males. Thus the haploid egg and the organism developing from it can indicate the effects of experimental treatment without the complications of another set of chromosomes. As will be detailed in "Materials and Methods," particular advantages were discovered in the use of Habrobracon for feeding experiments.

The purpose of the following experiments was to evaluate among embryonic offspring the total induced lethality, including recessive lethals, dominant lethals and non-nuclear damage, for a series of dosages ranging from tracer dilutions up into the intermediate category of radioactivity encountered in medical therapy (Levy, 1946). Phosphorus, the element chosen, is well represented in nucleic acids which are intimately associated with the structural elements of cell nuclei. Incidental observations on the longevity of treated animals are included, which indicate a remarkable radio-resistance of adult wasps.

MATERIALS AND METHODS

The Habrobracon stock used was wild type number 33 known at least 15 years for its high hatchability (Whiting, 1940). Unmated females were stored in clean shell vials (70 × 20 mm.) for five days in an incubator at 30° C. Such conditions

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of starvation produce a hungry individual with an abdomen very obviously flattened dorsoventrally. The visible change in abdominal shape reflects a utilization of reserves stored in the fat body and the disappearance of all but a few full-sized uterine eggs (Grosch, 1950). At the end of the fifth day a droplet of white clover honey with or without P^{32} was delivered to the wall of the vial from the end of a straightened No. 3 paper clip. Typically the hungry wasp is attracted to the honey within one minute, and, standing upon clean surface, contacts the honey only with the mouth parts. This is a distinct advantage over experimenting with insects which move over the surface of a radioactive medium when feeding. The gonads of an insect with such "browsing" habits are constantly near an external source of irradiation and the interpretation of results is thereby complicated. We believe that ingestion experiments with Habrobracon most nearly approach an evaluation of the effects of internal radiation alone. Note further that only one feeding was used per female.

During feeding, the flattened abdomen gradually swells until its fullness approaches that of an unstarved wasp. The re-attained rotundity is due entirely to the filling of an expansible crop situated in the anterior of the abdomen. This is a second advantage, because by mere external inspection the actuality and degree of feeding can be determined.

The braconid digestive tract extends in direct course from mouth to anus with the crop an enlarged, thin-walled connection between the esophagus and stomach. It is the structure known in the honey bee as the honey stomach. It is not a diverticulum as in other orders of insects and within a day its contents are moved into the midgut, yet the crop with its strong anterior and posterior valves can serve in efficient immediate storage of a significant volume of ingested food (Grosch, 1951). The capacity of the crop furnishes a defined volume basis for dosage in feeding experiments (a third advantage). The mean volume for distended crops from undwarfed adult females was determined as 0.49 ± 0.06 mm^3.

When the crop is completely filled, the wasp loses interest in feeding. The majority of Habrobracon females attain satiety at one continuous feeding although a few individuals seem to prefer intermittent ingestion. Because of the latter type of behavior, the standard procedure adopted was to allow a given female one hour in the vial with the food droplet. At the end of such a period the great majority of wasps are completely fed. This was determined by an inspection of the degree of distension of the abdomen.

Twelve hours after the single honey feeding each female was given two pre-stung caterpillars on which to deposit eggs. Stender dishes (60 × 35 mm.) large enough for spreading out the 10 caterpillars parasitized by 5 females were the containers used at this phase. Caterpillars rested upon white paper fitted into the bottom of the dishes. A daily renewal of one caterpillar per female has been adopted as the standard procedure. If additional caterpillars appeared discolored or unduly flaccid, they were replaced and a notation made on the data sheet. Such precautions are believed necessary to avoid introducing variables due to the condition of the host.

Eggs were collected every 24 hours and immersed in mineral oil (Nujol) contained in Syracuse watch glasses, a procedure determined as giving hatchability records even better than eggs left on the host. After 48 hours incubation in mineral oil at 30° C., the number of larvae which had hatched was counted and recorded. In our experience, since 30 hours is the normal hatching time at 30° C., all larvae which can emerge will have done so in 48 hours.

In all but the first experiment a weight basis was used for the preparation of samples of honey containing P^{32} at various activities. To this end, small (36 × 12 mm.), thick-walled bottles equipped with plastic screw caps were weighed before and after adding pipetted amounts of honey and diluted radioactive phosphorus in the approximate proportions needed to obtain a desired activity. In setting up the initial experiment it had been discovered that the forces of adhesion and cohesion negated pipetted delivery of exact volumes of honey. Dosages mentioned below are given in terms of the activity at the time of ingestion. The complicated question of internal dosage, although under consideration, has not progressed to a state which would warrant usage in the present paper.

Records upon the laying and hatchability were taken for 42,832 eggs, of which 24,832 were controls. Table I presents a numerical summary of experiments to indicate their scope and sample size. Also shown is the number of females used in each experiment. The analysis presented in the section on "Results" is a consideration on an average-per-animal basis.

TABLE I

A summary indicating the size of ovipositing groups and the total number of eggs laid by each group

Experiment number	Experimentals			Controls	
	Activity of material ingested in μc/g.	Number of females	Eggs laid	Number of females	Eggs laid
5	1,445	22	498	19	3,496
6	1,070	22	604	19	3,010
1	1,000−	19	769	21	3,379
2	271	17	816	18	2,377
3	166	20	2,671	20	3,152
4	43	16	2,471	17	1,820
7	20.6	20	2,264	17	2,684*
9	16.99	24	4,640	24	4,914
8	12.6	16	2,854	17	2,684*
		176	17,587	155	24,832

* The same control was used for two simultaneous experiments, numbers 7 and 8.

RESULTS

The gametic sequelae of treatment internally with P^{32} were found to have organization on the basis of the treatment's influence upon egg production. That is, egg laying (1) may or (2) may not be halted comparatively early in the life of a treated female. A dose adequate to halt egg production is about 200 μc/g. At lower doses, egg production continues throughout the life of the female. However, two types of effects are observed: (a) Between doses of 50 to 200 μc/g. there is definite falling off in egg production followed by recovery; while (b) below 50 μc/g. no statistically significant decrease in egg production is demonstrable.

(1) *Egg laying halted.* Figure 1 depicts the average day by day egg production for females fed sufficient radioactive phosphorus to cause early cessation of egg laying. Presumably at massive dosages, still fewer eggs would appear in an

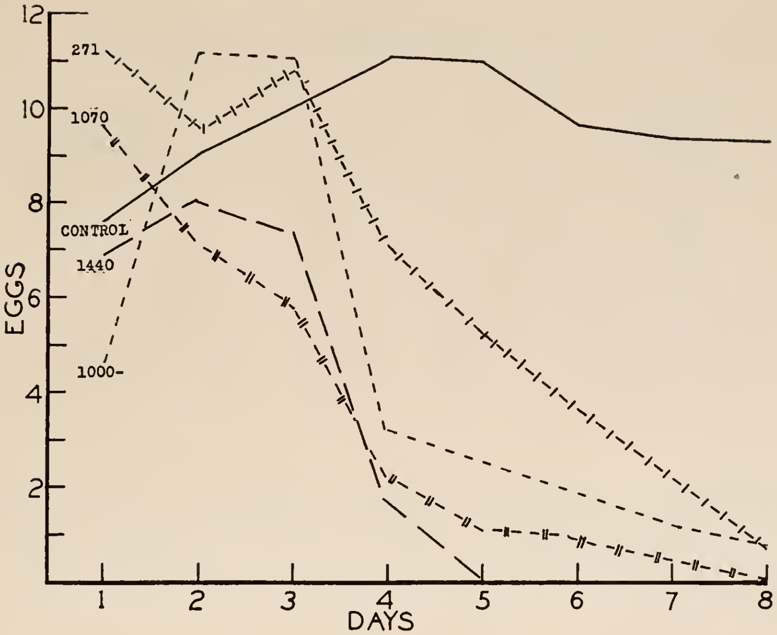

FIGURE 1. A comparison with combined controls of the average number of eggs produced per female per day by Habrobracon which ingested radioactive phosphorus sufficient to halt egg production. The low number of eggs produced in the first day of the 1000-experiment is non-significant; the individual control showed a similar value. The designation at the beginning of each curve is the activity in millicuries per gram of the mixture fed.

μc

approach to the limit of those eggs differentiated before treatment and un-resorbed during a five day starvation period. On the present graph an orderly relation is noted with the highest dosage causing earliest discontinuation of egg laying and the least number of eggs laid. At successively lower dosages a sequence in number

TABLE II

Cumulative total of average number of eggs produced per female

Experiment number	Activity of mixture ingested in μc/g.	Day 1	Day 4	Day 9	Day 19	Day 29	Day of death
5	1,445	7	23				23
6	1,070	9	26	28			28
1	1,000–	5	30	36			36
2	271	11	39	50			50
3	166	10	47	82	103	169	206
4	43	7	31	74	126	190	192
7	20.6	3	27	64	91	140	158
9	16.99	9	36	76	174	258	269
8	12.6	2	29	67	153	226	249
Controls	0	8	38	85	161	217	238

TABLE III

Average number of eggs produced per female during designated periods

Experiment number	Activity of material ingested in μc/g.	Days					
		1	2–4	5–9	10–19	20–29	29–death
5	1,445	7	16				
6	1,070	9	17	2			
1	1,000–	5	25	6			
2	271	11	28	11			
3	166	10	37	35	21	66	37
4	43	7	24	43	52	64	2
7	20.6	3	24	37	27	49	18
9	16.99	9	27	40	98	84	11
8	12.6	2	27	38	86	73	23
Controls	0	8	30	47	76	56	21

of eggs laid is seen corresponding to the dosage of radioactive phosphorus ingested. Tables II and III give a numerical synopsis of these facts.

On Figure 2 is plotted the percentage of the Figure 1 eggs which hatched. In general, the higher the dosage of ingested radioactive phosphorus, the lower is the hatchability. An inspection of Table IV, which summarizes hatchability in periods

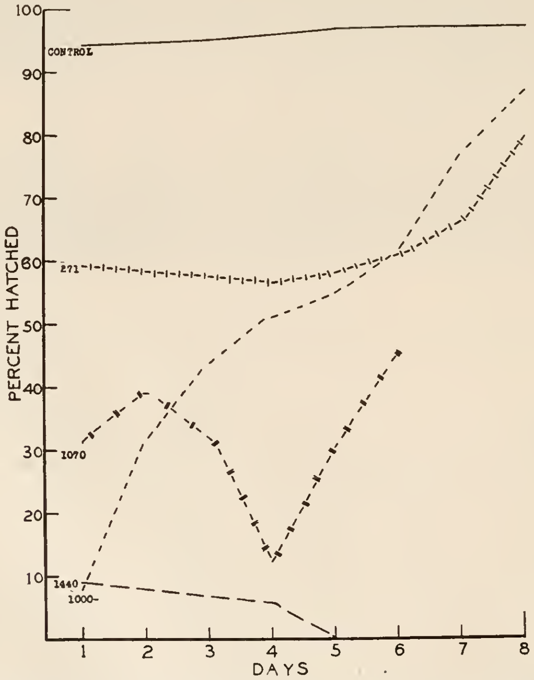

FIGURE 2. The hatchability of the eggs of Figure 1. The activity in millicuries per gram of the mixture fed is again indicated at the beginning of each curve.

TABLE IV

Average percentage of hatchability of eggs laid during designated periods

Experiment number	Activity of material ingested in μc/g.	Days					
		1	2–4	5–9	10–19	20–29	29–death
5	1,445	9	7				
6	1,070	9	22	28			
1	1,000−	8	44	67			
2	271	60	58	67			
3	166	93	87	85	52	53	63
4	43	98	97	96	95	80	50
7	20.6	51	83	84	75	57	45
9	16.99	96	97	96	94	87	—
8	12.6	59	91	94	91	80	62

selected on the basis of all primary curves (Figs. 1, 2, 3 and 4), demonstrates the hatchability relationship especially well in the 2 to 4 day period. In those experiments in which eggs were produced several days beyond the fourth day, there is shown a rise in hatchability for the last groups of eggs laid. However, it should

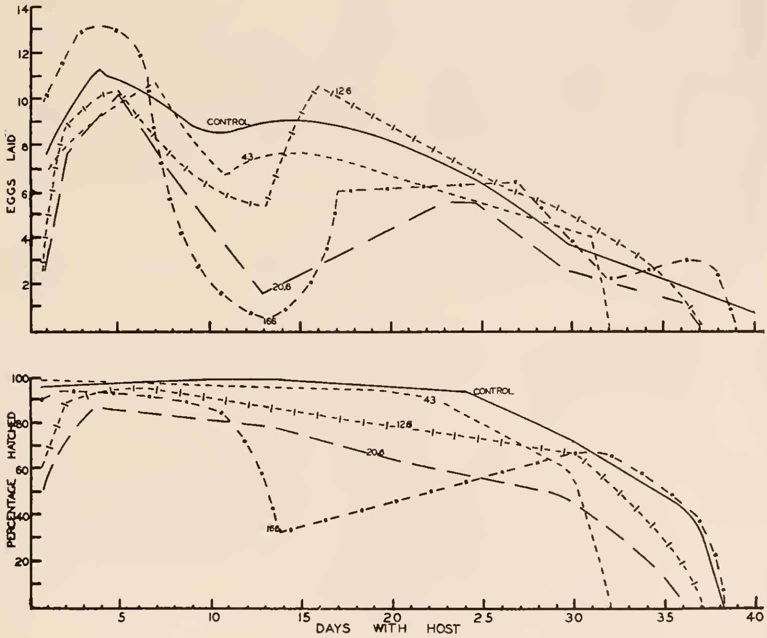

FIGURE 3. A comparison with combined controls of the eggs laid per female per day (above) and the hatchability thereof (below) in experiments when eggs are produced throughout the life of treated females. Curves are smoothed after the thirtieth day. The activity in millicuries per gram of the mixture fed is indicated along the middle of each curve where space is more adequate rather than at the beginning of the curve when the activity was at the level designated.

be remembered that these samples are quite small and the viability of even a single egg markedly affects the numerical result.

(2) *Egg laying not halted.* Figures 3 and 4 depict eggs laid and the hatchability thereof for experiments in which egg production continued throughout life. The results plotted in Figure 4 were treated separately to avoid adding confusion to already complex drawings (Fig. 3). Furthermore, unlike all other experiments, in the investigations for Figure 4, egg laying and hatchability were not recorded after the thirty-first day of oviposition. (In actuality, records beyond the thirty-first day would have been insignificant additions to the data obtained since only one animal survived the thirty-second day.)

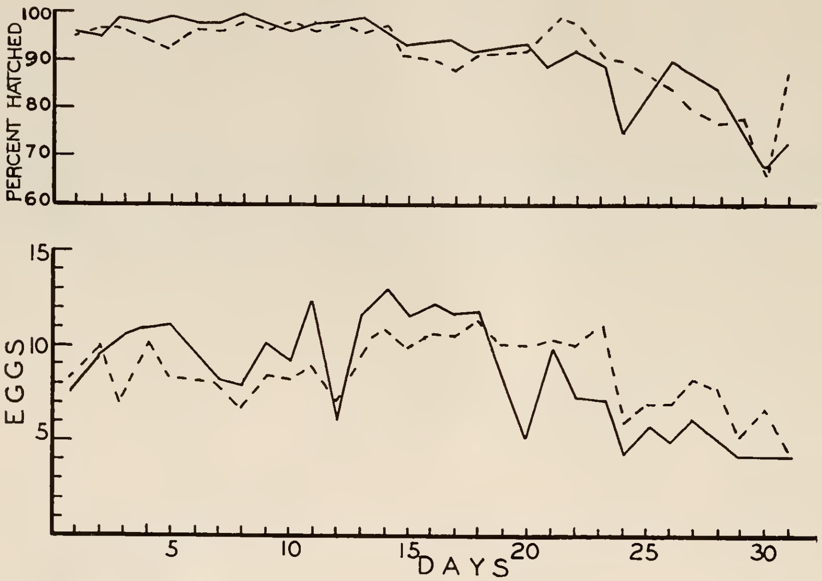

FIGURE 4. The eggs produced (below) and the hatchability thereof (above) in experiment No. 9. The solid lines represent control values. The broken lines represent results on an average per animal basis for feeding with a honey mixture at a radioactivity of 16.99 millicuries per gram.

In general, egg production both in controls and in experimentals is characterized by a peak between days 4 and 9 followed by a low between days 9 and 19. Following this low, the number of eggs laid per day becomes high again until about day 29. However, except for one experimental case to be discussed, egg production does not recover to the level of that during days 4 to 9. During the senescent period, the final days of life after the thirtieth day, there is a gradual dropping off in eggs produced.

As is shown in Table II higher doses tend to result in fewer eggs. When plotted graphically this consequence of treatment is not strikingly evident except throughout the course of days 9 to 19. During this period the drop in egg production is much accentuated in higher dosages. However, after day 19 the production of eggs by treated and untreated wasps becomes similar so that curves approach each other and differences are not significant. Table III furnishes a numerical summary by designated periods of eggs laid.

The results observed when the number of emerged larvae is compared with the number of eggs laid are rendered in percentages and plotted in Figures 3 and 4. The resultant curves are not as complex as those for egg production. Except for the highest dosage in this group (166 μc/g.) no obvious depression was demonstrated in the graphs.

In general, until day 29, the experimental curves parallel the curve for controls but at a lower value indicating a measure of lethality. After the twenty-ninth day the differences between controls and experimentals are statistically nonsignificant. In fact, in unsmoothed plots from raw data the lines cross and recross almost at daily intervals. Again it should be remembered that the samples of eggs obtained become small in the last few days.

Survival curves

A popular method of representing the results of radiation experiments is by means of the survival curves obtained through plotting the proportion of organisms surviving against the dose. This gives a picture of the manner in which the number of organisms affected increases with the dosage of radiation. In the present case, the values of the first three designated periods shown in Table IV have been used as a basis for Figure 5. The curves obtained promise to be particularly useful by indicating ranges of irradiation in which it would be interesting to have additional data. The curves indicate a more complicated type of action than the geometric progression (exponential curve) of a system involving single ionizations of targets (Lea, 1947). This was expected in view of the inclusive nature of induced lethality in the present experiments. On the other hand, it was interesting to find that our curves for first day egg hatchability exhibit a shape similar to that obtained when the emergence of adult Drosophila is plotted against the initial activity of P^{32} added to culture medium (Arnason, Irwin and Spinks, 1949).

Longevity

By calculating the means and their standard errors, a conception is obtained of the survival time of treated and untreated females. These results are presented in Table V, calculated as days after emergence from cocoons. To obtain days after

TABLE V

Mean longevity in days of females which have ingested P^{32}

Activity of material ingested in μc/g.	Experimental	Control
1,445	23.14 ± 2.08	27.89 ± 1.35
1,070	24.82 ± 1.74	25.37 ± 1.58
1,000–	21.32 ± 1.81	27.09 ± 2.47
271	25.06 ± 3.09	22.06 ± 2.31
166	26.45 ± 2.16	23.45 ± 2.02
43	26.94 ± 1.83	21.59 ± 2.11
20.6	29.10 ± 2.75	26.94 ± 2.71
16.99	27.17 ± 1.90	27.92 ± 1.85
12.6	28.63 ± 1.96	26.94 ± 2.71
	Combined controls	25.73 ± 0.65

treatment, five days should be subtracted from each value. It is evident from the data obtained that longevity of wasps was not influenced significantly by internal β radiation over the range investigated. In fact, the experimentals show higher mean survival time than the corresponding controls in five out of the nine experiments. In the distribution and range of values which make up the array from which mean survival time is calculated, examples of both treated and non-treated wasps were obtained in the long-lived category of 40 days after the honey feeding. Typically, at least three to six animals were alive after 30 days of experimental observation. Such longevity was spectacular in experiments in which egg production was halted five to eight days after treatment, yet the sterilized females remained alive for weeks after laying their last egg. A more comprehensive investigation into what appears to be remarkable radio-resistance by adult braconids is in progress.

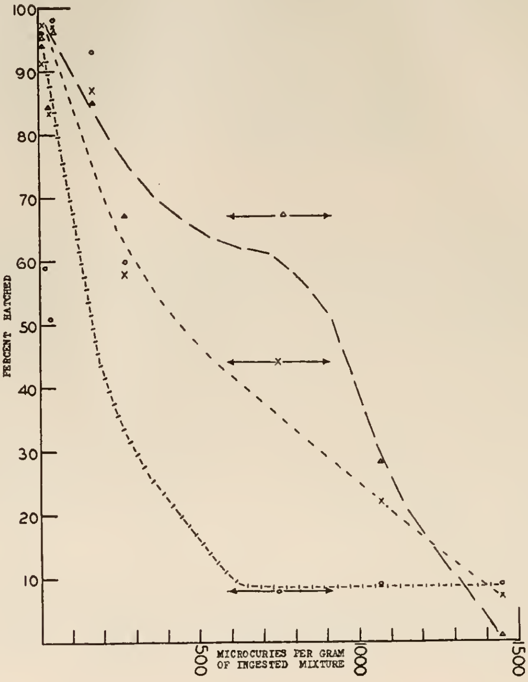

FIGURE 5. Survival curves obtained by plotting the percentage of hatched eggs against the radioactivity of the mixture ingested. The results for the first day of egg laying are designated by ovals. The corresponding curve is drawn of alternating horizontal and vertical dashes. The results for the period comprising the second to fourth day are designated by x marks and the curve is drawn using short dashes. The results of the period comprising the fifth to ninth day are designated with triangles and the curve is drawn using long dashes. The arrows mark the possible range of dosage for the initial experiment when pipetted measurement was attempted.

DISCUSSION

Interpretation of the above results is difficult because we have not been able to find comparable studies with the day-to-day life-time approach. In general the total and average egg productivity of controls compares well with the surveys of Hase (1922) and associates who obtained more than 200 eggs per individual braconid collected from nature. However, hatchability records by these European investigators run between 50 and 70 per cent whereas hatchability in the selected laboratory stock used by us and by A. R. Whiting is typically above 95 per cent. Even when the senile period is included the overall average hatchability is at least 80 per cent.

Occasional checks on egg production and hatchability at various ages have been made during exploratory and control studies by A. R. Whiting (personal communication). However these data have not been published nor do they include observations during the senile period. In the published studies with unmated females (Whiting, 1940, 1945) attention is directed chiefly to the first period of oviposition in the interest of comparing the radio-sensitivity of eggs in successive stages of first meiotic pro- and metaphase. Habrobracon furnishes ideal material for such studies and the results throw some light upon certain of our findings.

The braconid ovary is comprised of four ovarioles of the polytrophic type, thus containing alternating successions of oocytes and trophocytes for at least three quarters of each tube's length. The anterior end of each ovariole is made up of mitotically active oogonia subsequently differentiated into cell groups, of which the most posterior cell becomes the functional oocyte while remaining members of the group become trophocytes. As an oocyte is moved posteriorly its nucleus passes through prophase of first maturation division; eggs ready to be deposited are stored in the posterior of each ovariole in first meiotic metaphase. These observations from dissections were correlated with the results of X-radiation experiments (Whiting, 1940) to demonstrate that eggs in first meiotic metaphase were relatively susceptible to radiation damage, whereas oocytes in prophase with diffuse chromatin were relatively resistant. Radio-sensitivity and radio-resistance were reflected by low hatchability and high hatchability, respectively. Such differences in sensitivity could explain the rise in hatchability shown by the present curves during days 6 to 8 wherein the greater percentages hatched of the eggs laid on these days could reflect a resistance of prophase eggs to radiation.

It is not settled whether most of the radiation damage occurs shortly after ingestion or if injury occurs during an appreciable number of successive days. At present we lean toward the former, since it is suspected that much radioactive phosphorus is lost by incorporation into the eggs laid. Although radio-autography of Habrobracon has not yet been performed, it is well established in biological material that the deposition of P^{32} is heaviest in regions of the greatest metabolic and mitotic behavior associated with growth and differentiation. Typically, in the adult holometabolous insect, cell division and differentiation take place only in the gonads. Thus, Irwin, Spinks and Arnason (1950) find that Drosophila gonads continue to show very high P^{32} content in the adult when general growth of all other tissues has ceased. Consistent with these concepts is the present fact that with Habrobracon egg laying prolonged throughout life, experimental results in both egg production and in egg viability ultimately approach control values.

There are certain differences between our results and those of A. R. Whiting especially noticeable in the first day of egg laying on which the number of eggs obtained by us is consistently lower. This is undoubtedly due to the differences in the conditions of the experiment. The animals of the present experiment were maintained under circumstances which prevent the formation of uterine eggs, whereas the conditions in the Whiting experiments were conducive to the accumulation of uterine eggs.

Our finding a cessation of egg production after subjecting females to higher dosages of internal radiation seems another addition to the several reports of the radio-sensitivity of oogonia in various animals. Four examples have come to our attention: (1) With higher X-ray dosages Whiting (1940) discovered oogonial regions vacuolated, fragile and with pyknotic nuclei attendant to a falling-off in Habrobracon egg production. (2) A case of total absence of ovaries has been reported for female mosquitoes raised in baths containing P^{32} at activities of around 200 mμc. (Bugher and Taylor, 1949). (3) The most spectacular effect of internal radiations in vertebrates is shown in mice injected with P^{32} by reduction in the size of ovaries (Warren, MacMillan and Dixon, 1950). The immediate size reduction following injection is due to degenerative follicular changes; however, a failure to regain normal size is traceable to subsequent greatly diminished oogenesis. (4) Ionizing radiations are found to exert a selective and destructive action on the gonads of round worms (Alicata, 1951). These representative cases from such diverse phyla suggest a universal consequence to be expected in radiation experiments. It seems likely that numerous other examples may exist in published and unpublished data on still other types of animals.

When our observations on unhalted egg laying are considered, for comparison are available the lifetime records of egg laying for birds, especially chickens (Hutt, 1949), and for other insects, especially Drosophila (Gowen and Johnson, 1946). Records for all forms of life show a decline in egg production with age as do the present data. In this effect of senescence, as might be expected, the abilities of Habrobracon more nearly resemble those obtained for dipteran rather than those obtained for avian egg production. This is explainable on the basis that the normal egg production by insects is not markedly influenced by cyclic events. Egg laying as plotted by us for Habrobracon shows smoother day-to-day curves for the first half of the wasp life period than exhibited by eight different races of fruit flies considered by Gowen and Johnson. However, by suggesting two peaks, our curves seem more complicated in their general shape. A current experiment is being performed in an attempt to estimate whether starvation conditions preceding egg laying complicate the egg production curve. If not, an alternative explanation may be based on the change-over from eggs produced by oocytes differentiated before eclosion to eggs produced by oocytes differentiated during the laying period.

Lifetime records of modifications to egg laying, due to experimental effects comparable to ours, have not been discovered in available literature. In order to obtain data somewhat comparable, we have gone to reports concerning the effects of ionizing radiations on cell production, where we find curves which ours resemble. This is so for diagrammatic presentations of the rate of change in peripheral blood cells following graduated dosages of penetrating radiations (Cronkite, 1949). Blood cells are the ultimate products of mitotically active tissues. In turn, this thought suggested another source of comparable curves, those based upon mitotic index. Here, too, we note a resemblance, especially in the shapes of our lines plotted for the period between days 9 and 29 (Fig. 4). These curves are interestingly reminiscent of certain results obtained by Canti and Spear (1929) in irradiated, cultured chick tissue. Similar consequences of radiation have been demonstrated in other rapidly dividing tissues from such diverse origins as beans, rats and tadpoles (Lea, 1947). As measured by division activity, higher dosages result in a drop in cell production with the control level never regained, whereas in intermediate dosages a recovery wave is obtained which exceeds the control level. A present example of a recovery wave exceeding the control may be furnished by the 12.6 μc/g. curve.

An accepted explanation of such recovery waves is that the mode results when two classes of cells arrive at the same stage at a given time. A class of cells not in the susceptible phase at the height of treatment, and hence not greatly influenced, is supplemented by another class of cells which were delayed in division but have recovered. In explanation of our lifelong egg production curves we postulate similar delayed division effects upon the oogonia. It is conceivable that the oogonia forming a mitotically active cell mass would have cells in susceptible phase at the height of the treatment, which could be delayed in division and development.

SUMMARY

1. Changes in the eggs produced per day and in the hatchability thereof are reported for virgin Habrobracon fed honey containing P^{32} at radioactivities ranging from the tracer level into the intermediate level of internal therapy (10 μc to 1500 μc).

2. Above 200 μc/gm. of ingested mixture, egg laying ceased after a period of a few days corresponding to a fraction of the supply of unresorbed ova and oocytes differentiated before treatment. The viability of the embryos from these eggs, as measured by the number hatched, is correlated with the level of activity in the material ingested. A rise in viability in the seventh to eighth day, in experiments in which egg production continued that long, is interpreted as an indication of the lesser susceptibility of prophase eggs.

3. Below 200 μc/gm. of ingested mixture, egg laying continued throughout the life of the treated females; however, with higher dosages fewer eggs were produced. This is traceable chiefly to a decline in the eggs laid daily during the middle period of life. Curves obtained show recovery waves reminiscent of those found for cell production from rapidly dividing tissues after irradiation. Viability of the eggs produced during the first two-thirds of life is correlated with dosage. During the senescent decline in egg production, typical of both controls and experimentals, no significant differences in hatchability were obtained.

4. A remarkable radio-resistance was discovered, in that treated animals in *all* experiments lived as long as or longer than controls.

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REGENERATION IN ISOLATED AND FUSED PIECES OF CLAVA LEPTOSTYLA [1]

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Modern experimental studies on regeneration in hydroids, as Peebles (1931) anticipated, have been aimed at elucidating the factors responsible for this dramatic morphogenetic response. It is well known (reviews of Child, 1929, and Barth, 1940a) that coelenterate hydranths exhibit axial gradients of metabolic activity and that distal levels exert dominance over proximal levels. Barth (1938b) demonstrated that in Tubularia circulation within the gastrovascular cavity is responsible for the maintenance of dominance, for if an isolated stem was ligatured hydranths might regenerate at both ends. The same result was attained if circulation in the coelenteron was blocked with an oil droplet (Barth, 1938b), with a bubble of oxygen or nitrogen or by means of an inserted glass rod (Rose and Rose, 1941). A series of recent papers have considered certain extrinsic factors affecting regeneration. Temperature (Moog, 1941) and the level of oxygen supply (Barth, 1938a, 1940b, 1944; Goldin, 1942b; Miller, 1937; Rose and Rose, 1941) have been shown to be important in Tubularia. Accumulation of metabolic wastes (Rose and Rose, 1941; Miller, 1942) or lowered pH (Goldin, 1942a, 1942b) prevent regeneration. Zwilling (1939) showed that hydranths could be elicited along the side of a stem of Tubularia by cutting a window through the perisarc, thereby exposing the coenosarc directly to sea water, and Goldin and Barth (1941) have investigated the reorganization of coenosarcal fragments free of perisarc. As background for further work on the physiology of regeneration, the present paper deals with the potentialities for regeneration revealed in isolated or fused pieces of hydranths of *Clava leptostyla* Agassiz. Papers by Hargitt (1906 and 1911) contain valuable descriptive information on this species and a more recent paper by Brien (1943) reports experiments on regeneration in a related species, *Clava squamata*.

MATERIALS AND METHODS

During the summer of 1950 *Clava leptostyla* was found to be abundant on the Fucus attached to rocks in the intertidal zone at Salisbury Cove, Maine. Clava grows in the form of colonies of separate light orange hydranths (Fig. 1) attached to a mat of hydrorhiza firmly adherent to the substratum. Mature polyps, which are about 1 cm. in length when expanded, consist of a contractile stalk, a gonosome, tentacle-bearing region, and hypostome. The gonosome is a region possessing several short, branched gonophores bearing clusters of spherical sporosacs, which are either male or female. There is no free medusoid generation; instead a planula

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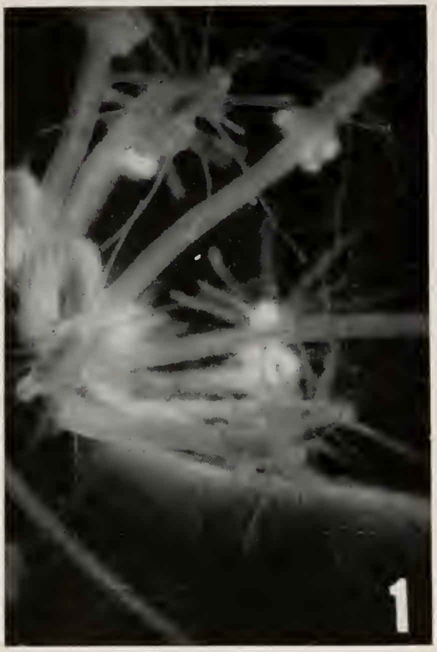
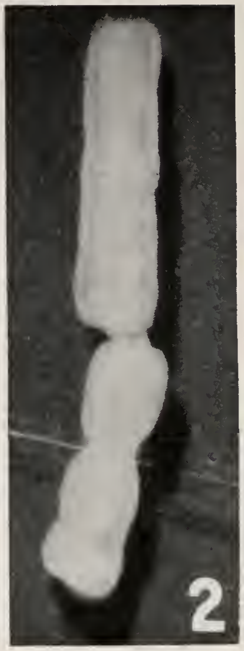
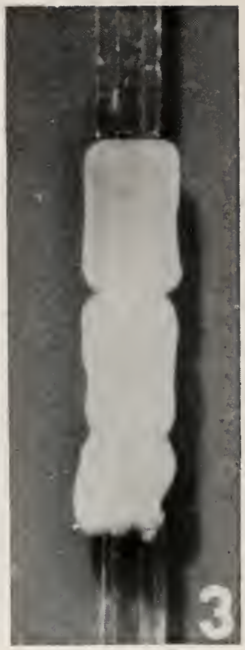
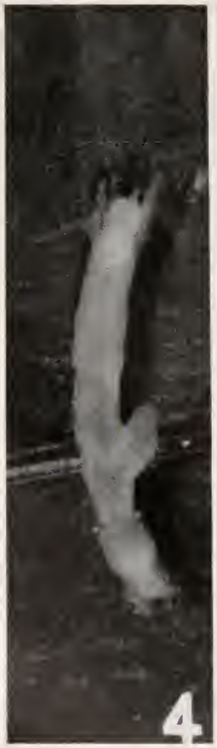
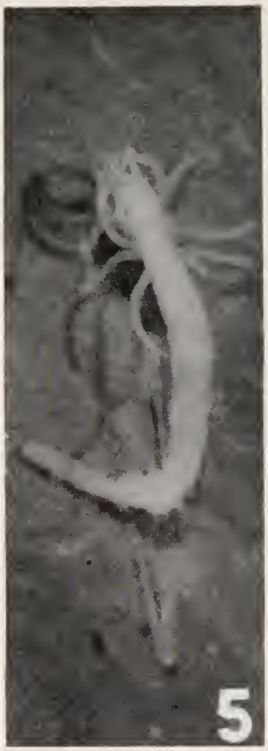
PLATE I

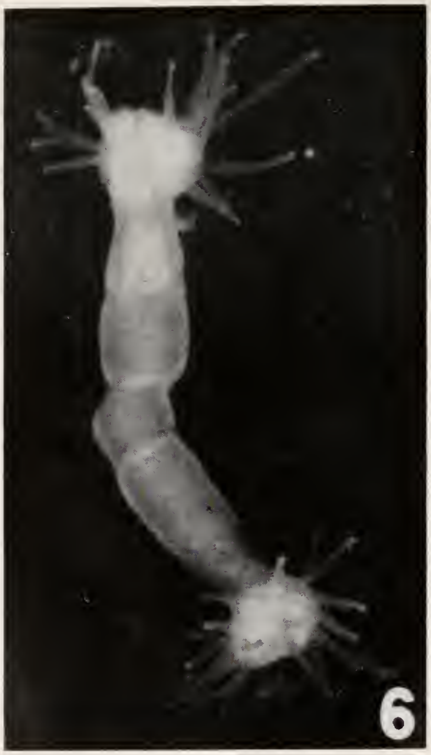












larva is released. There is no separate perisarc around the stalk of the hydranth but the perisarc is represented by a thin, non-living surface layer closely adherent to the ectoderm. Pieces to be isolated or fused were cut from individual hydranths with iridectomy scissors. Operations were performed in sea water and pieces were cultured at room temperature in small petri dishes immersed in finger bowls containing approximately one inch of filtered sea water. In early experiments it was noticed that isolated portions of the stalk usually rounded up and failed to undergo regeneration unless one end became attached to the culture dish. Accordingly, it became routine procedure to secure one end of an isolated piece by pinning it with a fine glass rod thrust through the tissue (Fig. 2) and into a substratum of 2% agar. Pieces to be fused were strung on a fine glass rod, then held tightly together for about 12 hours by means of glass tubing slipped over the ends of the rod and held in position with flat sections of cover glass resting on the exposed outer ends of the rod (Fig. 3). Photographs were taken with a Kodak Bantam camera mounted above one ocular of a stereoscopic microscope.

I wish to thank Dr. Maria Anna Rudzinska for directing my attention to the abundance of Clava at Salisbury Cove, and Dr. Philip R. White, in whose laboratory this work was done.

EXPERIMENTS

A. *Pieces isolated without agar substratum*

The first experiments tried were simple tests of the regenerative behavior of (1) the isolated hypostome and tentacle-bearing region, (2) the gonosome, (3) isolated stalks. Ten days after isolation 7 of the first group had become attached to the glass culture dish and were putting out hydrorhizal protuberances. Twenty-four others which remained unattached showed no basal outgrowths. It was noted by the twentieth day that most of the unattached specimens had undergone dedifferentiation of the tentacles originally present and had not regenerated. Those attached still showed their distal tentacles as well as a regenerated stalk and hydrorhiza. Isolated gonosomes (group 2) showed a marked tendency to regenerate new tentacles and hypostome at the apical end. After 10 days this was true in 8 of 23 specimens examined and one of these had regenerated two new oral ends. None

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FIGURE 1. View of colony of *Clava leptostyla* showing expanded hydranths; gonosome with spherical sporosacs just proximal to tentacle-bearing region.

FIGURE 2. Specimen illustrating method of pinning with a fine glass rod used to anchor stems to substratum of 2% agar. Here rod is through middle of one member of a fused pair of stalks.

FIGURE 3. Specimen illustrating method of fusing pieces. Two stalks strung on a single glass rod are being fused end-to-end through pressure exerted by glass tubes slipped over each end of the rod.

FIGURE 4. An unusual example showing regeneration of apical structures at both ends of a stalk isolated for 15 days. Hydrorhizal outgrowth is seen at level of glass rod through original basal end of stalk; basal hydranth possibly a result of secondary regeneration.

FIGURE 5. A typical example of primary regeneration showing tentacles at original apical end and hydrorhizum at original basal end of stalk isolated for 15 days; pinned through basal end.

FIGURE 6. Specimen showing stalk of one hydranth fused between basal ends of stalks of two other complete hydranths. After 19 days no regeneration had occurred at lines of fusion.

of the gonosomal pieces became attached, hence did not develop hydrorhiza. In the third group (isolation of the stalk) many of the specimens developed bulbular enlargements with asymmetrical protuberances at one end, presumably the primordia of hydrorhiza from the original aboral end. Fifteen specimens, all still unattached, had these outgrowths after 10 days, but only 7 of the 15 had regenerated new tentacles. By the twentieth day 4 had become attached. Two of these had regenerated oral structures at the apical end of the stalk and one had a new hydranth growing out from the basal hydrorhiza.

In another group of experiments the regenerative behavior of isolated individuals of Clava in various dilutions of sea water was tested. Dilutions differing by 10% intervals from 0 to 100% sea water were tried. Forty per cent sea water or less proved too hypotonic, but the organisms showed remarkable tolerance in surviving all dilutions down to 50% sea water. Two groups of complete hydranths, isolated in 50% and 80% sea water, respectively, survived during 17 days of observation. It appeared that during this time dedifferentiation of tentacles and gonosome was more rapid than in controls kept in 100% sea water. After dedifferentiation had occurred, regeneration of new hydranths took place. One specimen in 80% sea water had regenerated 8 new hydranths at the end of the 17 days.

The above results and others not reported here indicated that two types of regenerative response should be distinguished: (1) primary regeneration resulting in the differentiation of hypostome and tentacles at the apical end, usually within 3–7 days after isolating pieces of the hydranth; and (2) secondary regeneration, meaning the delayed differentiation of completely new hydranths either from hydrorhizal outgrowths or from dedifferentiated tissue of any part of an old hydranth. The former response undoubtedly depends on the apico-basal gradient already established, while the latter represents establishment of a new center of organization in an undifferentiated primordium. The results indicated also that the mechanical factor of attachment favors both primary and secondary regeneration.

B. *Isolated stalks pinned to substratum of agar*

After the importance of attachment was realized, a series of experiments on isolated stalks was performed. Isolated pieces were pinned with a fine glass rod through one end or through the middle. One end of the rod was then thrust vertically into a substratum of 2% agar in the culture dish. This procedure favored primary regeneration (Figs. 4, 5) since pieces anchored at one end could elongate. They were thus saved from rounding up and degenerating, the usual fate of unanchored stems. The results are recorded in Table I.

TABLE I

Primary regeneration of isolated entire stalks pinned to substratum

Position of pin	No. of animals	Age after isolation	Number regenerating	% regenerating
Apical end	10	7 days	1	10
Middle	19	7 days	12	63
Basal end	15	7 days	11	73

It is seen that there is a distinct difference between specimens pinned through the basal end or middle of entire stalks and those pinned through the apical end. Only 10% in the latter group showed primary regeneration, contrasted with 63–73% in the former groups. It appears that the apico-basal gradient is not changed when pinning is basal or central but that the tendency of the oral end to regenerate apical structures is inhibited by pinning through that end.

C. *Fusion of pieces*

It was possible by the technique described under Methods to fuse portions of animals in various combinations, including (1) apical ends together, (2) the apical end of one against the basal end of the other, (3) basal ends together, and (4) a portion of one animal between portions of two other animals (Fig. 6). The particular combinations tried are outlined in Table II.

TABLE II

Fusion of portions of hydranths

Type of fusion	No.	Age in days after fusion	Results
A. Two pieces			
1. Apical-to-apical			
a. Hypostomes removed; tentacular regions joined	1	13	No primary regeneration. Dedifferentiation of tentacular and gonosomal regions
b. Hypostomes and tentacular regions removed; gonosomes joined	6	13	No primary regeneration. Dedifferentiation of gonosomal region
*c. Stems only	15	7	No primary regeneration
*2. Apical-to-basal (stems only)	9	7	No primary regeneration
*3. Basal-to-basal (stems only)	20	7	No primary regeneration
B. Three pieces Pieces numbered below were fused between basal ends of stalks of 2 complete hydranths			
1. Hypostome	4	10	No primary regeneration. Incorporation into stems
2. Tentacular region	5	10	Extra tentacles in tentacular region; later dedifferentiation
3. Gonosomal region	7	12	No primary regeneration. Dedifferentiation of gonosome
4. Stem	5	19	No primary regeneration, no apparent dedifferentiation

* Pinned through middle of one member.

Several generalizations emerge from an examination of the results recorded in Table II. The first is that primary regeneration of parts excised did not take place when portions of hydranths were fused in apical juxtaposition (Experiment A-1). Primary regeneration could have occurred at the apical end if these parts had been isolated separately. This result illustrates the importance of environment in determining morphogenesis at the cut surfaces of hydranths. A similar general explanation would account for the results of fusing pieces between two other hydranths (Experiments B-1, B-2, B-3 and B-4). Although the grafted piece did undergo slight regeneration in one series (extra tentacles in Experiment B-2), generally it merely underwent dedifferentiation. There was no evidence of an inductive effect of the graft on the differentiation of adjacent regions of the host stems.

A third result is that obtained in the fusions of stems in apical-to-basal or basal-to-basal orientation. In these, either one (Experiment A-2) or two (Experiment A-3) apical ends were freely exposed to the culture medium. One member of the fused pair of stems was pinned through the middle to the substratum as in the simple experiments of isolation. In none of the fused pairs, however, was there any evidence of primary regeneration of tentacles at the free apical end.

Dedifferentiation of tentacular and gonosomal regions in Experiments A-1a and A-1b, B-2 and B-3 appeared to be a response to unfavorable physical and chemical conditions, perhaps comparable to those accounting for dedifferentiation of isolated single hydranths exposed to a hypotonic medium or failing to become attached to the substratum.

Discussion

The experiments reported in this paper have raised several interesting problems. First of all, we should like to understand the differences between what we have called "primary" and "secondary" regeneration. In primary regeneration the apico-basal gradient already established apparently is maintained, and a single new oral end develops within a few days at the apical end of a cut piece. Secondary regeneration depends upon a more profound dedifferentiation of existing structure or upon the outgrowth of hydrorhiza, and therefore takes several days longer than primary regeneration. Several new hydranths may develop in close proximity in secondary regeneration, indicating that the dedifferentiated region has become equipotential. These two types of regenerative response were also encountered by Goldin and Barth (1941) in their experiments on regeneration of expressed coenosarcal fragments of Tubularia as compared with stems retaining the perisarc, and by Brien (1943) in *Clava squamata*. Further investigations, both histological and physiological, are required to reveal how the cells actually behave during both primary and secondary regeneration.

A second problem is to explain why regeneration is favored by attachment to the substratum. Normally, of course, Clava is attached basally and may elongate or contract. An isolated stem continues to exhibit these movements, but it frequently rounds up after a few hours, ceases its motility and after several days degenerates without any sign of regeneration. Possibly apical and basal ends in rounded specimens are brought so closely together that the apico-basal gradient is abolished; perhaps internal pressure inhibits differentiation of new oral structures. Attachment by pinning obviously provides the stem with a practical substitute for its normal attachment, thereby enabling elongation and contraction, conditions which appear to be favorable for primary regeneration. As for secondary regeneration, attachment is a *sine qua non.* Hydrorhiza must have a substratum for continued outgrowth and dedifferentiated pieces need to be attached in order to put out new hydranths.

Two additional problems are raised by the inhibition of primary regeneration in (1) isolated stems pinned through the apical end or (2) stems fused together. In the pinning experiments it may be guessed that the glass rod mechanically changed the environment of the apical cells so that they could not move to the positions usually taken in regeneration or could not divide so readily. Another possible explanation is that the apical end pinned close to the substratum was deprived of oxygen or exposed to accumulated waste products in such concentration that regeneration was inhibited. Experiments on the effect of pinning on the respiratory metabolism of Clava might help resolve this problem. Inhibition by fusion is not unexpected in the experiments of joining two pieces with apical ends together or fusing pieces between two other stems. Cells which, if exposed to the culture medium, would form part of regenerated oral structures would in fusion be joined with neighboring cells and mechanically prevented from organizing into a regenerate. More difficult to explain, however, is the inhibition of primary regeneration in stems united in apical-to-basal or basal-to-basal orientation. The data presented in Table II are too few to give any clear idea of the mechanism of inhibition. The long (12 hours) process of fusion on a glass rod may have altered the stems in some manner inimical to regeneration. Unfortunately, the control experiment of holding a single stem on a glass rod for up to 12 hours before culturing was not tried. It is possible that under the conditions of the experiments there was an oxygen deficiency. The experiments ought to be repeated with larger numbers of animals and with particular attention to environmental conditions known to favor regeneration, namely, pH, temperature, oxygen and removal of waste products.

SUMMARY

1. Observations have been made on regeneration of isolated pieces of hydranths of *Clava leptostyla,* including the hypostome and tentacle-bearing region, the gonosome and the stalk.

2. Stalks isolated in 50% and 80% sea water underwent accelerated dedifferentiation as compared with controls in normal sea water.

3. The distinction is drawn between (1) "primary" regeneration, meaning the differentiation of missing oral or basal ends under the influence of the existing apico-basal gradient and (2) "secondary" regeneration, the delayed development of new hydranths from attached hydrorhiza or dedifferentiated tissue.

4. Primary regeneration at the apical end of isolated stalks usually occurred after anchoring the stem against 2% agar with a fine glass rod thrust through the middle or the basal end. Regeneration was inhibited, however, if the apical end was pinned.

5. Primary regeneration of apical ends was repressed by fusion of pieces of 2 hydranths in apical-to-apical juxtaposition or by fusion of an apical piece between the basal ends of two other hydranths.

6. Apical regeneration likewise failed to occur in fusions of two stalks in apical-to-basal or basal-to-basal orientation.

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AGGLUTINATION OF FISH AND TURTLE ERYTHROCYTES BY VIRUSES[1]

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Following the observation by Hirst (1941) that influenza viruses agglutinate chicken erythrocytes, it was found that several other viruses are capable of agglutinating erythrocytes from certain animal species (Burnet, 1942; Lush, 1943; Levens and Enders, 1945; Nagler, 1942; Burnet and Boake, 1946; Mills and Dochez, 1944; Hallauer, 1949; Lahelle and Horsfall, 1949; Olitsky and Yager, 1949; Sabin and Buescher, 1950). Moreover, it was demonstrated that anti- or immune serum against the corresponding virus inhibits hemagglutination by the agent. As a result, the hemagglutination reaction became a much used technique for measuring either viral or antibody concentration and is useful also in the serologic diagnosis of a number of viral diseases. The reaction of influenza, mumps, and Newcastle disease viruses with erythrocytes proved of special interest when it became evident that these viruses are able to elute spontaneously from erythrocytes with which they combine (Hirst, 1942). As the virus elutes, the erythrocytes lose their capacity to adsorb fresh virus. This loss is accompanied by a reduction in the electrophoretic mobility of the erythrocytes (Hanig, 1948; Ada and Stone, 1950; Stone and Ada, 1950). The eluted virus, however, is capable of combining with and agglutinating fresh erythrocytes (Hirst, 1942). A considerable amount of evidence has accumulated favoring the view that influenza, mumps, and Newcastle disease viruses are capable of effecting an enzymatic alteration of receptor areas on the erythrocyte surface. It appears probable that the receptors are mucoprotein in nature. It should be emphasized that none of these viruses is capable of multiplying in erythrocytes. What role such enzymatic activity has in the series of events leading from virus-host cell combination to multiplication of viruses in and their subsequent release from host cells is not established despite interesting work bearing on this problem (Stone, 1948a; 1948b).

The present study was undertaken in order to extend the range of observations in respect to erythrocyte-virus interaction. Erythrocytes from certain lower classes of vertebrates were employed. In addition to viruses known to cause agglutination of erythrocytes from higher classes of vertebrates, other viruses which have not been reported to agglutinate erythrocytes were studied.

MATERIALS AND METHODS

Viruses. Ten-day old embryonated chicken eggs were inoculated allantoically with PR8 or FM1 strain of influenza A virus, Lee or MB strain of influenza B virus, swine influenza, or Newcastle disease virus. The eggs were incubated at 35° C. for

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[1] Part of the work was carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts.

48 hours, then chilled at 4° C. overnight, and the allantoic fluid harvested. Habel or Enders strain of mumps virus was inoculated in 7-day old chicken embryos and allantoic fluid harvested 5 days later. The allantoic fluids were dialyzed against 80 volumes of 0.1 M phosphate buffer at pH 7.1, centrifuged at 3800 g for 10 minutes and the supernatants stored at 4° C. In addition, Lee virus was dialyzed against 0.85 per cent NaCl. Herpes simplex virus, K strain, kindly made available by Dr. E. D. Kilbourne, was inoculated on the chorio-allantoic membrane of 10-day old chick embryos. The membranes were harvested after incubation of the eggs at 35° C. for two days. A 50 per cent suspension was then prepared and homogenized in a modified Waring blendor. The suspension was clarified by centrifugation at 3800 g for 10 minutes and the supernatant stored at 4° C. Semliki Forest virus was inoculated allantoically in 10-day old chick embryos. The eggs were incubated for 30 hours. Allantoic fluid was harvested, dialyzed, centrifuged and stored as described above. The infected embryos, too, were collected and a 50 per cent suspension prepared and stored as described above. Columbia MM strain of mouse encephalomyelitis virus was inoculated intracerebrally in 5-week old white mice. The mice were killed two days later and a 25 per cent suspension of infected brain was prepared as described above. Coxsackie virus (Conn. No. 5 strain) was inoculated in one-day old suckling mice. The mice were killed four days later. A 10 per cent suspension of the carcasses was prepared, homogenized and centrifuged at 3800 g for 30 minutes. The supernatants were stored at 4° C. Pneumonia virus of mice (PVM) was inoculated intranasally in three-week old mice. The mice were killed 6 days later and the lungs collected. A 10 per cent suspension in water was prepared, homogenized, heated at 70° C. for 30 minutes, and clarified by centrifugation at 3800 g for 10 minutes. The supernatant was collected and stored at 4° C. Uninfected control suspensions of each of the various host tissues used were prepared.

In experiments with erythrocytes, untreated viruses or viruses heated at 56° C. for 30 minutes were employed as described below.

Erythrocytes. Blood, obtained with syringe and needle, was mixed with $\frac{1}{6}$ volume of a 2.5 per cent solution of sodium citrate. Two or more representatives of each species of animals were bled and their RBC tested. Erythrocytes were washed three times with appropriate diluent (see below) and a 1 per cent suspension prepared in the same diluent. It was found that erythrocytes from dogfish and turtle sedimented more rapidly in the centrifuge than those from mackerel, sea robin or tautog.

Dogfish (*Mustelus canis*). Blood was obtained from the caudal vein. The freezing point depression of blood of marine elasmobranchii is − 1.93 (Smith, 1931) which corresponds to the freezing point depression given by a 3.26 per cent solution of NaCl (Roth and Scheel, 1923). Because of the high concentration of urea in the blood, the diluent chosen for dogfish erythrocytes contained 1.64 per cent NaCl and 2.16 per cent urea (Lutz, 1930). Other salts were omitted.

Mackerel (*Scomber scombrus*), *sea robin* (*Prionotus carolinus*), *tautog* (*Tautogo onitis*). Blood was obtained by cardiac puncture. The freezing point depression of blood of typical marine teleosts is − 0.8 (Smith, 1931). Correspondingly, a 1.37 per cent solution of NaCl was used as the suspending agent (Roth and Scheel, 1923).

Turtle (*Pseudemys elegans*). Blood was obtained by cardiac puncture through a drill hole in the plastron. On the basis of freezing point depressions reported for various turtles (Botazzi, 1908; Burian, 1910), a 0.86 per cent solution of NaCl was used as the medium for turtle erythrocytes.

Hemagglutination tests. Serial twofold or tenfold dilutions of virus were prepared in 0.4 cc. volumes in the appropriate diluent and 0.4 cc. of 1 per cent erythrocytes added per tube (final concentration 0.5 per cent). The tubes were maintained at 25 or 5° C. for periods of time varying from 10 to 80 minutes, depending on speed of sedimentation and disagglutination of erythrocytes, as indicated below. The tests then were read. The last tube showing definite (2 + to 3 +) agglutination was taken as the end point. Titers are expressed as the reciprocal of the dilution of virus at the end point.

EXPERIMENTAL

Agglutination of fish and turtle erythrocytes by viruses. In the initial experiments with erythrocytes and viruses employed in this study, hemagglutination tests were carried out at 25 and 5° C. with three final concentrations of virus representing 1 : 2, 1 : 20 and 1 : 200 diluted allantoic fluid, mouse lung or mouse brain suspension. Table I summarizes the results of these experiments with various strains of influenza, mumps and Newcastle disease viruses. As can be seen, hemagglutination was observed in the great majority of tests. However, striking specificity was shown with PR8, FM1 and Lee strains of influenza virus which failed to agglutinate mackerel and tautog erythrocytes either at 25 or 5° C.

TABLE I

Agglutination of marine erythrocytes by influenza, mumps and Newcastle disease viruses

Erythrocytes	Virus							
	Influenza A		Influenza B		Swine influenza	Mumps		Newcastle disease
	PR8	FM1	Lee	MB		Enders	Habel	
Dogfish	+*	+	+	+	+	+	+	+
Sea robin	+	+	+	+	+	+	+	+
Mackerel	−†	−	−	+	+	+	+	+
Tautog	+	+	+	+	+	+	+	+
Turtle	−	−	−	+	+	+	+	+

* Plus sign indicates that definite hemagglutination was present at a dilution of 1:20 or higher.

† Minus sign indicates that no evidence of hemagglutination was present at a dilution of 1:2. Because qualitatively identical results were obtained at 25 and 5° C., each sign represents results recorded at both temperatures.

No agglutination of dogfish, mackerel, sea robin, tautog or turtle erythrocytes was observed under identical conditions with the following viruses: herpes simplex, Semliki Forest, Columbia MM strain of mouse encephalomyelitis, Coxsackie (Conn. No. 5) and pneumonia virus of mice (PVM).

Control experiments with normal allantoic fluid, suspensions of normal mouse lung and mouse brain gave no evidence of agglutination.

Comparison of hemagglutination titers. Lee and MB strains of influenza B virus were employed in comparative experiments with various erythrocytes. It was desired to determine the range of hemagglutination titers at two temperatures: 25 and 5° C. In addition, hemagglutination titers with viruses heated at 56° C. for 30 minutes were measured.

Table II gives the results of representative experiments of this kind. As can be seen, the hemagglutination titers at 25° C. fall within a range of 256 to 4026, with the exception of low titers obtained with Lee virus when sea robin erythrocytes were used and with MB virus and mackerel red blood cells. In the latter instances the titers observed at 60 minutes were lower than those at 30 minutes in contrast to the stable values observed with other virus-erythrocyte combinations within the time interval indicated.

The hemagglutination titers obtained at 5° C. were within the same high range as, or in certain instances somewhat higher than, the titers obtained at 25° C. In particular, Lee virus and sea robin erythrocytes as well as MB virus and mackerel erythrocytes gave high titers at this temperature.

TABLE II

Agglutination of marine erythrocytes by Lee and MB strains of influenza B virus

Treatment of virus	Temp. of agglut.		Lee virus					MB virus				
		Reading	Dogfish	Mackerel	Sea robin	Tautog	Turtle	Dogfish	Mackerel	Sea robin	Tautog	Turtle
	° C.											
None	25	1st*	1,024	0	16	1,024	0	2,048	64	256	512	256
		2nd†	1,024	0	4	1,024	0	4,096	8	256	512	256
56° C., 30′	25	1st	1,024	0	64	1,024	0	4,096	128	128	256	128
		2nd	1,024	0	64	1,024	0	4,096	128	128	256	256
None	5	1st	2,048	0	512	1,024	0	4,096	256	512	512	512
		2nd	2,048	0	512	1,024	0	4,096	256	512	512	512

* 1st reading at 20 minutes with dogfish and turtle erythrocytes, and at 30 minutes with mackerel, sea robin and tautog erythrocytes.

† 2nd reading at 40 minutes with dogfish and turtle erythrocytes, and at 60 minutes with mackerel, sea robin and tautog erythrocytes.

Hemagglutination titers obtained at 25° C. with heated viruses (56° C., 30 minutes) were, in general, closely similar to those observed with unheated agents at this temperature. However, heated Lee virus and sea robin erythrocytes, as well as heated MB virus and mackerel erythrocytes, gave titers somewhat higher than those observed with unheated viruses at this temperature. Moreover, the titers observed were stable, in contrast to those obtained with untreated viruses at this temperature. It should be noted that the titers observed with these two viruses (untreated) at 5° C. were still higher.

Disagglutination of erythrocytes. In view of the evidence that elution of influenza viruses from erythrocytes is accompanied by loss of the ability of such erythrocytes to agglutinate with the same virus (Hirst, 1942), experiments were done to determine whether elution of the virus and disagglutination or dispersion of agglutinated erythrocytes were correlated in time.

The Lee and MB strains of influenza B virus employed were dialyzed against 0.85 per cent NaCl and 0.1 *M* phosphate buffer, respectively. Chicken erythrocytes washed with and suspended in 0.85 per cent NaCl were used. Preliminary experiments were done to determine the most suitable proportions of virus and erythrocytes. If too high relative concentrations of virus are used, the considerable amounts of uncombined virus in the system interfere with measurement of the quantities of virus adsorbed or eluted per unit of time. On the other hand, if too high relative concentrations of erythrocytes are used, the large number of unagglutinated erythrocytes interferes with the counting of disagglutinated cells. These experiments showed that satisfactory adsorption-elution and agglutination-disagglutination curves could be obtained when undiluted allantoic fluid (with a hemagglutination titer of 1024) was mixed with an equal volume of 10 per cent erythrocytes.

Preliminary experiments were also done to determine the optimal temperature for elution, and with Lee virus 14° C. was chosen, whereas with MB virus elution curves were determined at 37° C. This difference indicates that Lee virus has a much faster rate of elution from chicken erythroyctes than MB virus.

The adsorption-agglutination and elution-disagglutination experiments were done as follows: Virus preparation and erythrocyte suspension were mixed at 4° C. in a 25 × 110 mm. test tube with a side arm. Homogeneous distribution of agglutinated and unagglutinated erythrocytes in the mixture was maintained by mechanical stirring at a fixed rate. At intervals, aliquots were removed with a pipette via the side arm. The erythrocytes in a part of each aliquot were promptly sedimented by centrifugation. The supernatant was collected and the concentration of virus determined by means of hemagglutination titration. The other portion of the aliquot was diluted 1 : 5 with 0.85 per cent NaCl and unagglutinated erythrocytes were counted in a hemocytometer. After a suitable period of time, the reaction mixture was brought to the temperature at which elution-disagglutination could be followed optimally and additional aliquots were removed and examined as described above.

Figure 1 gives the results of representative experiments with Lee and MB viruses. As can be seen, elution of virus and disagglutination of erythrocytes followed essentially parallel courses.

In experiments with erythrocytes from marine species, a large number of instances of spontaneous disagglutination of erythrocytes was observed. The results with Lee virus and sea robin erythrocytes and with MB virus and mackerel erythrocytes were discussed above. In addition, similar evidence was obtained with dogfish, tautog and turtle erythrocytes. These experiments were carried out at 25° C. The period of observation was prolonged to four hours by re-suspension of sedimented erythrocytes by shaking at regular intervals. Disagglutination of erythrocytes from each of the five species employed was observed with two or more influenza virus strains and with Newcastle disease virus. Agglutination of erythrocytes by mumps virus was followed by spontaneous disagglutination in the case of turtle, mackerel and dogfish erythrocytes.

In different virus-erythrocyte systems, loss of agglutinability appeared after strikingly different periods of time. The rate of action of Newcastle disease and Lee viruses on dogfish and sea robin erythrocytes was found to be considerably faster than that of other viruses employed, including PR8, FM1, MB, swine and mumps. Moreover, marked differences were noted in respect to the time required by different erythrocytes to lose their agglutinability when mixed with one and the same virus,

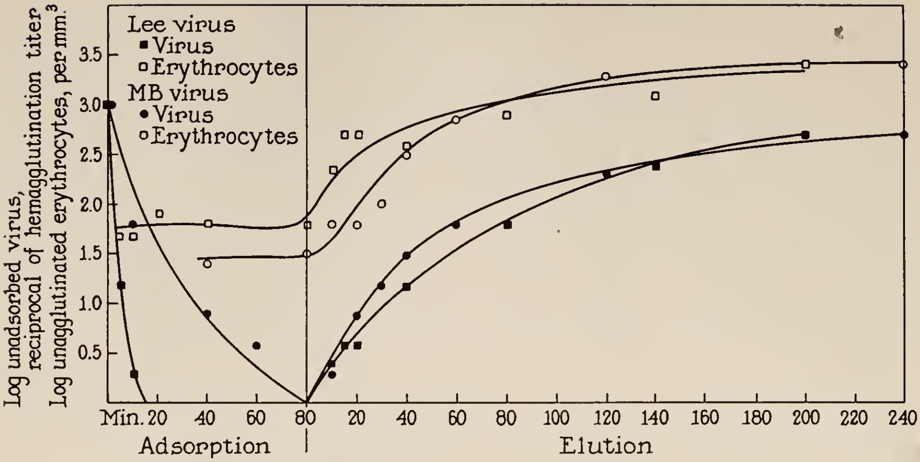

FIGURE 1. Relation of adsorption and elution of virus to agglutination and disagglutination of erythrocytes. With both Lee and MB viruses, adsorption was carried out at 4° C. Elution was permitted to take place at 14° C. with Lee virus, and at 37° C. with MB virus. In both experiments the control virus titer was 1024. Chicken erythrocytes, final concentration 5 per cent, were used.

e.g., employing MB virus, longer periods were required with tautog, sea robin and dogfish erythrocytes than with mackerel or turtle cells. In no case was evidence of disagglutination observed when tests were done at 4° C.

It appeared, therefore, that disagglutination was a regular phenomenon and that the exceptional instances where disagglutination was not observed may have been due to experimental conditions used: if temperatures higher than 25° C. and periods longer than 4 hours had been employed, disagglutination might have occurred in such instances.

DISCUSSION

The finding that influenza, mumps and Newcastle disease viruses are capable of agglutinating erythrocytes from teleosts and elasmobranchii is of interest because it focuses attention on the extremely broad species range of this reaction: the agglutination of reptilian, amphibian, avian and mammalian erythrocytes by representatives of this group of viruses has been demonstrated previously (Smadel, 1948). Studies with chicken erythrocytes have yielded suggestive evidence that in the hemagglutination reaction influenza, mumps and Newcastle disease viruses combine with receptor groups on erythrocytes which are mucoprotein in nature (de Burgh *et al.*, 1948; Hirst, 1948). Strong, although indirect, support for this contention has come from studies with mucoid components of tissues and body fluids which react with influenza, mumps and Newcastle disease viruses (Tamm and Horsfall, 1952). One of these components, a mucoprotein present in normal human urine, has been shown, following isolation and purification, to be a single homogeneous substance by electrophoretic and ultracentrifugal criteria (Perlmann *et al.*, 1952; Bugher, 1951). The features which characterize virus-erythrocyte interaction also characterize that between mucoprotein from urine and influenza, mumps or Newcastle disease viruses:

combination between the reactants is followed by emergence of free virus and demonstrable alteration in the mucoprotein. The altered mucoprotein is not able to combine with virus and its electrophoretic mobility is reduced (Perlmann *et al.,* 1952). As in the case of virus-erythrocyte interaction, the rate at which the virus causes alteration of the mucoprotein is considerably faster with certain viruses than with others. For example, the rate of Lee or Newcastle disease virus action on the mucoprotein is more rapid than that of MB, FM1, mumps or swine influenza viruses (Tamm and Horsfall, 1952). The findings reported in this study are in agreement with these observations.

Thus, it appears probable that erythrocytes from species ranging from man to the dogfish possess similar surface receptor areas with which influenza, mumps or Newcastle disease viruses are able to combine, and which they alter during the process of elution. However, within the species range of virus-erythrocyte interaction, instances can be found in which agglutination does not take place, as illustrated by the failure of PR8, FM1 or Lee viruses to agglutinate mackerel or turtle erythrocytes. Structural fit as well as adsorptive forces may be factors involved in this phenomenon for which at present an explanation is lacking.

In contrast to the group of viruses discussed above, pneumonia virus of mice, the various mouse encephalomyelitis viruses, and Japanese B encephalitis virus are agents which agglutinate erythrocytes from only a very few animal species. Agglutination of erythrocytes by these viruses takes place under conditions which are different in each case. These agents do not show spontaneous elution and nothing definite is known about the chemical nature of the groupings on the red cell surface with which these viruses combine. It should be emphasized that numerous viruses have failed to give the hemagglutination reaction with any erythrocytes against which they were tested.

SUMMARY

1. Dogfish, sea robin or tautog erythrocytes are agglutinable by all strains of influenza, mumps or Newcastle disease viruses employed.

2. Mackerel and turtle erythrocytes are agglutinable by mumps or Newcastle disease viruses as well as by some strains of influenza B virus. In the presence of these agents, agglutinable erythrocytes lose their capacity to be agglutinated by the same virus and spontaneous elution of the agent occurs. Elution of virus from erythrocytes and disagglutination appear to occur concurrently.

3. Among a number of other viruses tested, none was capable of agglutinating erythrocytes employed in this study.

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RESPIRATORY STUDIES OF SINGLE CELLS. I. METHODS[1]

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For a detailed study of respiratory events in single cells, even large ones such as the commonly used marine eggs or protozoans, a stability of a few $\mu\mu$l/hour[2] or better and a sensitivity down to a fraction of a $\mu\mu$l are desirable. Only one method (Zeuthen, 1943) has been described so far that approaches this order of magnitude. By modifying and reducing drastically the conventional Cartesian diver respirometer (Linderstrøm-Lang, 1937, 1942, 1943; Linderstrøm-Lang and Glick, 1938; Boell, Needham and Rogers, 1939; Holter, 1943), he was able to achieve a stability of 0–20 $\mu\mu$l/hour and a sensitivity of about 20 $\mu\mu$l. With this 0.1 μl diver he measured the oxygen consumption of a single polychaete worm egg and of single amoebae. In another series the method was used in a study of the single, half, whole and syncytial egg of an ascidian (Andresen, Holter and Zeuthen, 1944; Holter and Zeuthen, 1944).

Large single amphibian eggs have been studied by means of larger and less sensitive modifications of the conventional Cartesian diver (Zeuthen, 1946).

In this paper we present a method for micro respiratory studies of single cells that so far has yielded results with a sensitivity of about 0.2 $\mu\mu$l and a stability (drift) from 0–10 $\mu\mu$l per hour. The method is in some respects simpler than the 0.1 μl Cartesian diver and seems to offer possibilities for further refinements. It is to be hoped it will prove its usefulness among flotation methods for measuring respiration, the development of which has been so successfully carried out at the Carlsberg Laboratory.

I. THE RESPIROMETER

In 1911 Krogh developed a method for micro gas analysis which he named microscopical gas analysis and which consisted essentially of measuring the diameter of gas bubbles under the microscope before and after absorption of the CO_2 and O_2. In 1941, it seemed to one of the authors that it might be possible to place a cell in a small chamber full of water together with a bubble of oxygen. The chamber would be in pressure equilibration with the outside water bath by means of a fine capillary connecting space. The cell would consume the bubble and the rate of shrinkage could be measured through the microscope. The stability of a bubble in such a system without a cell was tested, using a chamber identical with the ones used at present, and was found very good. However, as the bubble shrinks from the oxygen consumption the surface tension builds up an increasing gas pressure in it, according to the relation $P = \frac{4T}{D}$, where T = the

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[1] This work was supported by a grant from the William F. Milton Fund.

[2] One $\mu\mu$l is defined as one millionth of one cubic millimeter = liter × 10^{-12}.

surface tension and D the diameter. If T is taken as 73 dynes/cm., the pressure in the bubble due to the surface tension will be $\frac{2200}{D}$ mm. Hg when D is measured in microns. Hence, a 10 μ bubble has an overpressure of 220 mm. Hg, a 100 μ bubble an overpressure of 22 mm. Hg, etc. It is therefore clear that this principle can only be applied on a manometric basis where the pressure is so adjusted that the bubble volume is kept constant. As in any manometric method, the change in pressure will reflect the oxygen consumption when the volume of the gas and liquid content of the chamber are known. The problem therefore became how to know most accurately that the bubble was being maintained at constant volume. By microscopic measurement of the bubble the volume could be estimated with an accuracy of no more than three per cent, which was not very satisfactory. A much more sensitive way of ascertaining the volume constancy of our bubble would be to make use of the extreme accuracy whereby the density of minute objects can be determined by flotation (Linderstrøm-Lang and Lanz, 1938). The problem would then be to stick the bubble in the respiration chamber onto a hydrophobic minute weight and then to regulate the pressure so that the bubble would balance the weight suspended in the water.

In the Cartesian diver the constancy of the gas volume of the chamber is ascertained by floating the whole chamber. In the present system a minute reference diver is floated in the respiratory chamber containing the cell. The gas phase is less than one per cent of the water phase, and the pressure effect by respiratory CO_2 can, at most, amount to about three per cent of the changes in the oxygen tension, even in acid media. This is due to the much higher solubility of CO_2 than oxygen. At slight alkalinity the CO_2 pressure will remain practically zero. Usually, therefore, no special provision for CO_2 absorption need be made.

A. *Principle of the method*

A minute respiration chamber is filled with a medium which communicates to the same medium on the outside through a fine capillary space. This space serves as a diffusion barrier, but permits free pressure transmission to the chamber from the outside medium, upon which controlled pressure can be applied. The respiration chamber contains, besides the cell, a little bubble adhering to a minute hydrophobic weight. The pressure is adjusted so that the bubble always just suspends the weight to a constant height in the respiratory chamber. We have then a constant volume system, where the pressure changes, read on the manometer, multiplied by a factor calculated from known constants give the oxygen consumption. The CO_2 is effectively held in solution by the large liquid to gas ratio of the system and amounts to three per cent or less of the factor.

B. *Construction* (*Fig. 1*)

The apparatus is simple and can be assembled in almost any laboratory from conventional equipment. It consists of the respiratory chamber assembly with the reference diver, accessories for making the reference diver and for charging the respirometer, the manometer with pressure control, the water bath, and optical equipment.

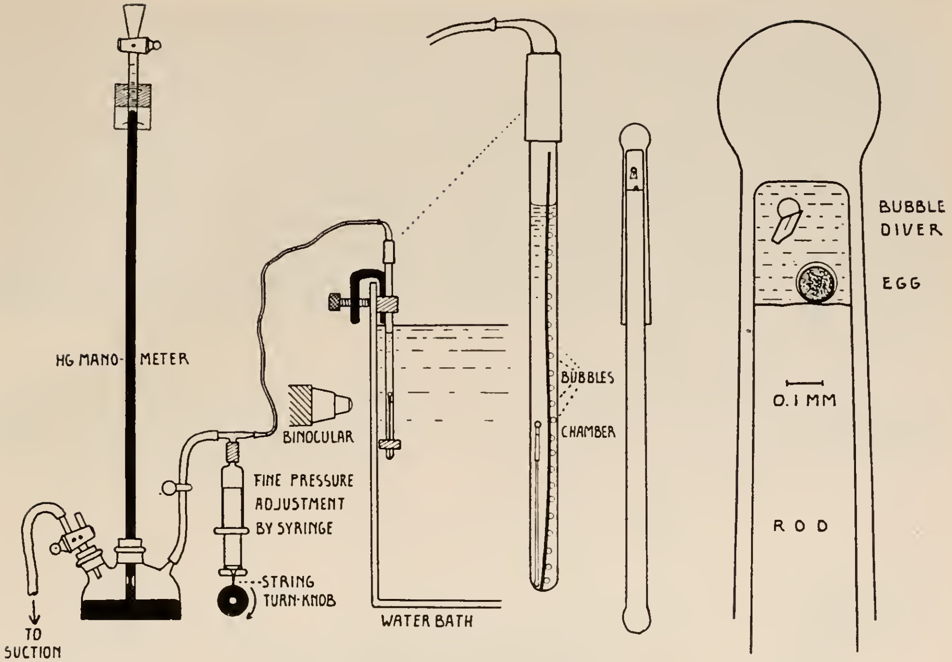

FIGURE 1. Respiration measurement of single cell by means of reference diver.

1. *Respiratory chamber assembly*

This consists of the chamber, the chamber tube, and the plastic reference diver.

a. Preparation of glass tubing. The respiration chamber is made from gently heated, soft glass.[3] We have not been able to use cleaning solution or detergents in the finished chambers as this would often produce drastic toxic effects on Paramecium, even after lengthy boiling and rinsing in water. All tubing is therefore cleaned before making the chamber. The roundest possible (check by micrometer) soft glass tubing is selected (2.5–3 mm. OD), and is cut into 50 or so pieces 10 cm. long. They are then put in a beaker with 2% Dreft (or other detergent), boiled for a few minutes and left covered by the same solution for future use.

b. Making the respiration chamber (Fig. 2). A soft glass rod, 2–3 mm. thick and as round as possible, is heated evenly by rotation in a flame and pulled out straight to form a symmetrical cone with a long taper, reaching the desired diameter of about 0.3 mm. while still tapered. The desired diameter is determined by a micrometer and the rod cut off at that point, with the least possible chipping. We have found a triangular India honestone to be a better glass cutter for these dimensions than a sharp Carboloy steel edge (which cuts the finger better than the glass). The thick end is cut off and flamed to a knob.

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[3] It was found that in the dimensions here used, a freshly made respiration chamber of Pyrex glass is highly toxic to some cells, Paramecium, for instance.

A glass tube is removed from the detergent solution and rinsed first under the faucet and then with distilled water. The outside is dried and the inside water is shaken out. The tube is rotated and heated evenly and pulled out to a long taper. The wide end is cut off and the rod stuck into it as far as it will go. It is essential to check under the dissecting microscope to see *that colored Newton rings are visible at the end of the rod all around the circumference extending a millimeter or more back from the end.* This is the only criterion of tightness and hence of the efficiency of the diffusion barrier between the chamber and the outside. It is usually necessary to try several glass tubes before a satisfactory fit is obtained. It is useless to try rods or tubing unless they are pulled out straight and form a symmetrical cone at the thick end. Unless these conditions are fulfilled the taper will not be round.

The rod is stuck rather loosely into the tubing, which is melted off in a micro flame[4] a few millimeters from the rod end (Fig. 2). In this way combustion products from the flame cannot enter the chamber. With the chamber still loosely stuck onto the rod, the sealed end is rotated and melted to form a symmetrical knob. The rod is pressed in carefully and the chamber is measured. The length should be 1–1.5 times the diameter. If it is too long loosen the rod and heat the knob again, until the desired length of chamber is obtained. The sleeve of the chamber is cut off at a total length of about 5–6 mm. The chamber is now ready for use.

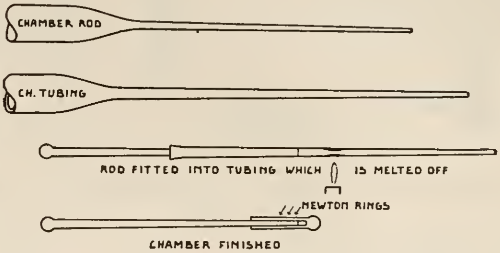

FIGURE 2. Making the respiration chamber.

c. The chamber tube (Fig. 1). The respiratory chamber rests during the experiment in a chamber tube about 0.5 × 12 cm., which is fastened to a clamp by means of a section of rubber tubing, so that it can be moved in all directions. The clamp screws onto the front of the water bath. A fine polyethylene tubing, 1.5–2 mm. OD, connects the tube with the pressure system. The tube also holds a tension equilibrating device, consisting of a piece of Saran filament, 1.5–2 mm. thick, pitted every 3 mm. with a sharp needle point. When the pressure is lowered to generate the bubble on the diver, bubbles will also form at each pit and this will facilitate the establishment of the same gas tension in the water outside and inside the respiration chamber.

d. The reference diver (Fig. 3). A piece of Saran tubing,[5] 10 cm. long and about 5 mm. OD, is rotated and very gently heated 10 cm. or so above a low Bunsen flame. When carefully done the tubing will turn clear and melt together and form a thinner capillary with a fine hole. One end is quickly clamped and the other end pulled out and stretched to a length of several meters, forming a fine filament. A piece 50–60 μ thick with a 1–2 μ hole is selected from the filament. It is wound on a card for safe keeping. While on the card the filaments are measured under a microscope and a piece 2–3 cm. long is cut out, having a uniform, accurately known diameter and a small central hole. The piece is fastened at one end by Scotch tape to a piece of a white plastic millimeter ruler attached to the top of a number 10 rubber stopper, which serves as a convenient "working table." A splinter of a new xylene-cleaned razor blade is twisted off (Fig. 3) and serves as a knife. Before cutting the diver under the binocular a little distilled water is run down on the filament and wiped off with clean gauze.

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[4] S. S. White (Philadelphia, Pa.) Orthodontic Burner No. 6.

[5] Manufactured by the Dow Chemical Co., Midland, Michigan.

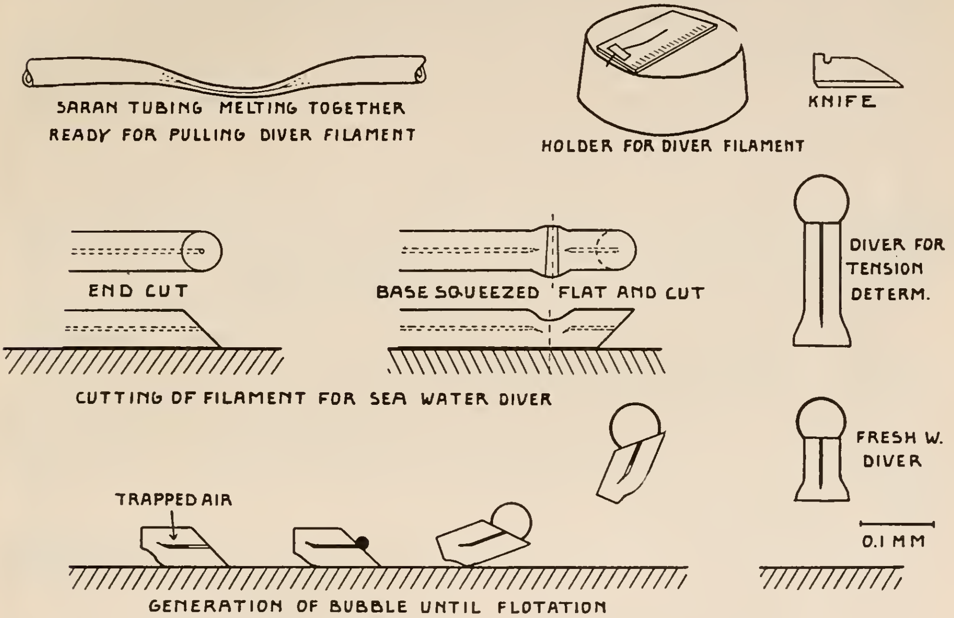
SARAN TUBING MELTING TOGETHER
READY FOR PULLING DIVER FILAMENT
HOLDER FOR DIVER FILAMENT
KNIFE
END CUT
BASE SQUEEZED FLAT AND CUT
DIVER FOR TENSION DETERM.
CUTTING OF FILAMENT FOR SEA WATER DIVER
TRAPPED AIR
FRESH W. DIVER
0.1 MM
GENERATION OF BUBBLE UNTIL FLOTATION

FIGURE 3. Preparing reference diver and bringing it to flotation.

We have used two types of reference divers. One has the top cut obliquely to prevent the bubble from hitting the chamber wall, which is hydrophobic in sea water. The other type has a straight cut top and can usually be used in fresh water, which wets the chamber walls.

To cut the oblique diver, the holder is placed under the binocular and the filament held down with a washed and dry finger. The end is cut with the very tip of the razor blade, slanting it at a 45° angle against the table. The diver filament must be seen to have air in the central hole where it is cut. The cut must be clean and sharp, leaving the hole open. Roll the filament over 180° and squeeze the base of the diver flat with the back side of the knife point, so that it is seen that the capillary closes. Then cut straight. The minute diver usually adheres to the knife point and is transferred directly into the already loaded respiration chamber. It can also be stored for some time in a drop of water without losing the air that is trapped in the capillary. It can then be transferred somewhat more readily from the drop by means of a braking pipette (Holter, 1943; Claff, 1947) or micrometer burette provided with flexible plastic micro tubing ending in a fine glass tip.

A straight cut diver for fresh water or tension work is basically made the same way.

2. *Manometer and pressure control* (*Fig. 1*)

For the pressure measurements we have employed a closed mercury manometer of the same type as used in the Van Slyke-Neill manometric blood gas apparatus. With oxygen-charged water it gives a useful scale of about 600 millimeters. This type of manometer obviates the need for thermobarometer control and possesses a large pressure range, which seems desirable for this method.

The manometer is prepared as follows.[6] Suction is applied to the top, and the mercury is brought all the way up by slanting the manometer. A little ethylene glycol is run down in the tube on top of the mercury. Most of it is removed again by tilting and applying suction from the top. With the top stopcock closed the barometer is evacuated by the suction pump[7] to get rid of the gases on the walls of the barometer tubing and in the ethylene glycol. The ethylene glycol is allowed to collect at the top and is carefully removed by suction from the top of the tilted manometer. It is necessary to get rid of most of it or it will collect on the meniscus and interfere with the reading.

Check that the barometer reads the correct barometric pressure by measuring the distance between the top meniscus and the lower surface in the mercury container. It should check within one or two mm.

Accurate reading of the barometer is greatly facilitated by the following simple device (Fig. 1). A white card with black backing at the top is clipped onto the barometer tube. For a reading the card is slid so that the black backing runs slightly above the meniscus. The meniscus will then stand out sharp black against a white background.

The pressure has one coarse and one fine adjustment. The coarse adjustment is provided either by applying vacuum from a steadily running vacuum pump or admitting air pressure by turning the two-way stopcock.

Fine adjustment is provided by the 5 cc. syringe arrangement shown in Figure 1. The syringe is kept lubricated by applying heavy oil around the barrel seal. The plunger must move smoothly without sticking.

3. *Water bath*

An ordinary large (40 liter) glass aquarium tank is used as water bath. It is insulated by Celotex or several layers of corrugated paper, except for the upper six or seven centimeters front and back. In front of the bath rests a horizontal binocular, preferably with rack and pinion up and down, and in back of the bath opposite the microscope a small fluorescent lamp. The water bath must be kept within ±0.01° C. or better of the desired temperature. If an automatic thermoregulator is used, it is imperative to check the magnitude of the cycling on a Beckmann thermometer during an hour's time to be sure that it does not exceed

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[6] For more detailed description see Peters and Van Slyke, 1932, p. 273.

[7] A laboratory suction-blower unit will do.

0.01 degree. The heating and cooling sources should be as gentle as possible. An infrared lamp suspended above the water bath and connected with a rheostat makes an excellent source of heat, and a mere trickle of cool water on the stirring propeller may serve as a cold source. By regulating the rheostat the temperature can easily be controlled within the desired limits, even by hand.

4. *Optical equipment*

For observing the cell and the reference diver, any type of binocular dissecting microscope mounted horizontally can be used. It must be provided with an ocular micrometer scale which can be calibrated against the grid of a blood counting chamber or even against a millimeter ruler stuck into the water bath. From 40 up to 100 times magnification has been found satisfactory.

For accurate measurement of the diver filament, egg sizes, etc., an ordinary microscope with an ocular micrometer is necessary. Another dissecting microscope is used for loading the chamber, cutting the reference diver, etc., and it is convenient to have it also provided with an ocular micrometer.

II. THEORY

The system is in principle a simple constant volume apparatus, where the constancy of the gas volume, as in a Cartesian diver, is determined by flotation rather than by reading a meniscus, but where the CO_2 is held in the medium rather than being separately absorbed.

A. *Equations and constants*

The oxygen consumption or CO_2 evolution is given by equations 1, 2 and 3, when only one gas is considered.

The following symbols will be used: V_B = volume of gas bubble; V_L = volume of liquid; P = observed pressure; W = water vapor tension; N = nitrogen tension; S = bubble pressure produced by surface tension;[8] T = absolute temperature; ΔP = observed pressure change; α = solubility coefficient for the gas that changes; F = the chamber constant. STPD means "measured dry at standard temperature and pressure."

Gas volume (STPD) at start is

$$V_B \frac{273}{T}\left(\frac{P-(W+N-S)}{760}\right) + V_L\alpha\left(\frac{P-(W+N-S)}{760}\right) = A. \quad (1)$$

Gas volume (STPD) at end is

$$V_B \frac{273}{T}\left(\frac{P-(W+N-S)-\Delta P}{760}\right) + V_L\alpha\left(\frac{P-(W+N-S)-\Delta P}{760}\right) = B. \quad (2)$$

$$\text{Gas consumed (or produced)} = A - B = \Delta P\left(\frac{V_B \frac{273}{T} + V_L\alpha}{760}\right), \quad (3)$$

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[8] See page 157.

which is the well-known formula for calculating the flask constant in a Warburg manometric apparatus.

In a system where CO_2 is not separately absorbed, as in our system, an expression for the oxygen consumption ΔO_2 in terms of the pressure reading ΔP is given by the following equations:

$$\Delta V_{O_2} = \Delta P_{O_2}\left(\frac{V_B\frac{273}{T} + V_L\alpha}{760}\right), \tag{4}$$

$$\Delta V_{CO_2} = \Delta P_{CO_2}\left(\frac{V_B\frac{273}{T} + V_L\alpha}{760}\right). \tag{5}$$

Assuming $RQ = 1$,

$$\Delta V_{O_2} = \Delta V_{CO_2}, \tag{6}$$

$$\Delta P = \Delta P_{O_2} - \Delta P_{CO_2}. \tag{7}$$

Solved for ΔV_{O_2} the final equation gives the oxygen consumption:

$$\Delta V_{O_2} = \Delta P\left(\frac{1}{\dfrac{760}{V_B\frac{273}{T} + V_L\alpha_{O_2}} - \dfrac{760}{V_B\frac{273}{T} + V_L\alpha_{CO_2}}}\right) = \Delta P \times F. \tag{8}$$

It will be noted that pressures which remain constant, such as those contributed by water vapor, nitrogen and surface tension of the bubble, cancel out in equations 1 and 2 and are of no influence as long as they remain constant. (See derivation of equation 3 in Dixon, 1943.)

Besides the pressure difference ΔP read on the manometer, we must know T, V_B, V_L, α_{O_2}, and α_{CO_2}. T is equal to the water bath temperature. V_B is very small and can be estimated closely enough from its measurement in the ocular eyepiece. It can be better estimated by knowing the volume per millimeter of the diver filament and calculating the volume of the diver from its measured length. With a specific gravity of Saran of 1.7, the bubble volume will be the diver volume times 0.7.

V_L is obtained from measuring in the binocular the diameter and the height of the chamber, allowing for the rounded upper corners. In order to save repeated calculations a chart is prepared, where on a log log paper the diameter from 0.1–1 mm. is plotted against the volume per millimeter height, which at 0.2 mm. diameter is 314,000 $\mu\mu$l and at 0.8 is 503,000 $\mu\mu$l. A straight line is drawn through these points. The volume/mm. read on the graph multiplied by the height in millimeters gives the chamber volume in $\mu\mu$l. The cell may conveniently and without significant error be considered water. The diver volume plus the bubble volume is usually smaller than the error in the estimation of the chamber volume and can then be ignored. Usually, therefore, V_L can be taken equal to the chamber volume.

The absorption coefficient of oxygen, α_{O_2}, for fresh water may be found in any handbook, and for sea water of different salinities and temperatures it may be found by interpolation of data given in Sverdrup, Johnson and Fleming (1942).

The absorption coefficient at different temperatures for carbon dioxide (α_{CO_2}) in acid media can be found in standard handbooks and for acid sea water in the above mentioned book. In neutral or alkaline media the CO_2 retention can without much difficulty be determined, as described below.

B. The pressure effect of CO_2 production

From equations 1 and 2 it can easily be shown that the ratio $\frac{\Delta P_{O_2}}{\Delta P_{CO_2}}$ depends upon the ratio of the solubility coefficients and the ratio of the liquid volume to the gas volume. In an all gas phase the carbon dioxide will of course exercise its full pressure, so that $\frac{\Delta P_{O_2}}{\Delta P_{CO_2}} = 1$, whereas in an all liquid phase the ratio becomes $\frac{\Delta P_{O_2}}{\Delta P_{CO_2}} = \frac{\alpha_{CO_2}}{\alpha_{O_2}}$. From Figure 4 it will be seen that when the gas volume gets smaller than 1% of the liquid volume, the pressure effect of the CO_2 gets to be very near to the lowest possible, namely $P_{O_2} \times \frac{\alpha_{O_2}}{\alpha_{CO_2}}$. In a system like this one we must therefore strive to keep the gas volume less than 1% of the liquid volume. In most of our work it has been 0.5% or less.

With an increasing alkalinity the pressure effect of CO_2 will naturally get smaller and smaller. Exactly what it will be can be determined in the following way.

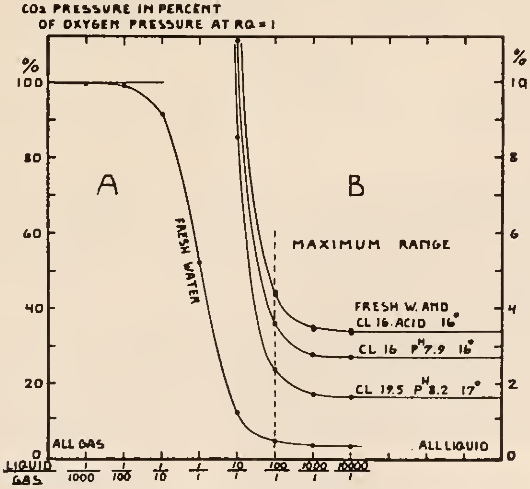

FIGURE 4. Pressure effect of carbon dioxide production in per cent of the pressure effect of an equal oxygen consumption when the gas-to-liquid ratio in the respirometer is varied. A. The entire curve for fresh water with ordinate units to the left. B. The lower part of three curves magnified ten times, with ordinate units to the right.

1. Determination of the CO_2 pressure

a. Principle. Increasing, known amounts of CO_2 gas are added anaerobically to a series of syringes containing 10 cc. sea water each. The CO_2 is dissolved quantitatively by chilling the syringes. The charged water is transferred with negligible gas loss to a long-necked 1 cc. flask in an accurately regulated water bath (Fig. 5). A micro reference diver is placed in the flask which is connected to a manometer and pressure regulating device (the same as for the respirometer chamber). The diver is brought to flotation and the pressure is read. This represents the total gas pressure plus the surface tension effect. A few cubic millimeters of strong NaOH are added from a microburette to make the sea water alkaline. The reference diver is again brought to flotation. The difference in pressure gives the CO_2 effect directly in mm. Hg.

b. Details of procedure. The flask is provided with a spiral depression so it can be vigorously stirred by twisting back and forth while still connected with the manometer. The diver is long so that the bubble will completely occupy the upper surface and hence will assume the same radius after collapse. Flotation pressure is established in a few minutes and read. The chamber is twisted to effect stirring and flotation pressure is read again until it attains constancy. The burette tip is conveniently made from a pulled-out polyethylene micro tubing. It must be very fine (0.1–0.2 mm.). For 0.7 cc. sea water, 1.50 $mm.^3$ 1 *N* NaOH sufficed.

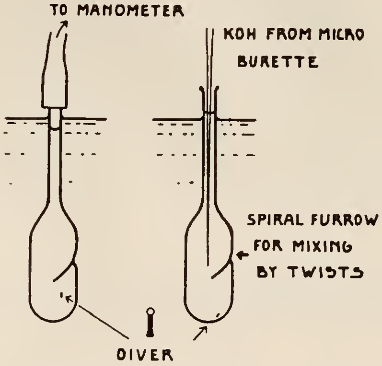

FIGURE 5. Reference diver technique for direct determination of CO_2 tension in liquid.

The action of NaOH upon the surface tension of water has been found to amount to a change from 72.8 to 74.6 dynes/cm. at 20° at a concentration of 5% (Landolt-Börnstein, 1923, p. 240). The amount of NaOH added to the sea water gives approximately 0.01%, and hence the effect from change of surface tension is negligible.

c. Results. The pressure effect of CO_2 was determined for the different sea waters which were used (Fig. 6). Carbon dioxide has greater pressure effect in acid sea water than in fresh water, because the salinity lowers the CO_2 solubility. The pressure effect decreases rapidly with increasing pH. At pH 8.2–8.4 it is only half or less that of fresh water. From the curves the average retention of the CO_2 can be calculated and hence the pressure effect of CO_2 for different gas-to-liquid ratios at different pH and salinity can be estimated (Fig. 4).

It will be seen that in all our experiments with sea water in the chamber, the pressure effect of the CO_2 has only amounted to 2–3% of the oxygen pressure effect. This correction is included in equation 8, but can usually be ignored because the chamber volume, and certainly the volume or weight of the respiring material, are known with less accuracy.

It is, however, important to know the magnitude of the CO_2 effect for the interpretation of any irregularities or bumps on the curves, and it is then well to realize that a maximum metabolic change in *RQ* from 0.7 to 1 will only show up as a 1% or less error in the oxygen consumption. Similarly, acid formation must be at least equivalent to the oxygen consumption on a molar basis before it begins to show up. In other words, the CO_2 binding properties of straight unbuffered sea water, such as used in our experiments, hold the CO_2 pressure effect down to a negligible amount. With less favorable gas-to-liquid ratio, and for special purposes, it may, however, be desirable to add a buffer to the sea water (Zeuthen, 1950).

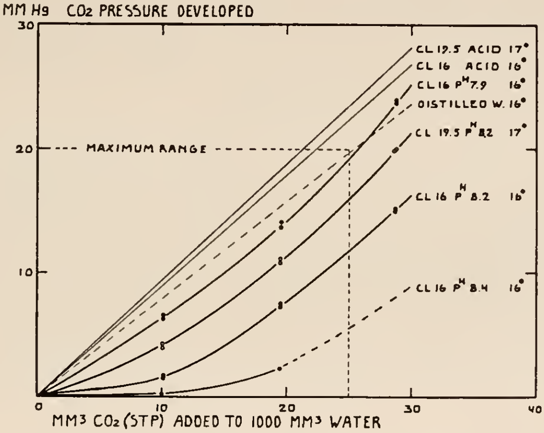

FIGURE 6. Pressure developed by adding known amounts of CO_2 to fresh water and sea water of varying pH and chlorinity.

The maximum amount of oxygen that can be used up in a chamber containing oxygen equilibrated water corresponds to some 2–3% of the water volume. The maximum CO_2 that can be metabolically produced at *RQ* 1 is then the same, namely around 20–30 volumes per 1000 water. The pressure effect of this can be read on Figure 6, and corresponds to 20 mm. Hg. It will usually be less than 10 mm. or corresponding to about 1% CO_2 at atmospheric pressure.

C. *Diffusion characteristics of the system*

This system, like all others, depends upon diffusion equilibrium between the gas and the water phase. The faster equilibrium is regained after a disturbance, the faster the system will be able to follow quick changes in respiration. Since the diffusion distances are very short in all directions (maximum 0.3 mm.) and the volumes involved are very small, there is an exceptionally rapid tension equilibrium within the system.

This can easily be determined empirically. A blank is set up without respiring material and a 10–15 minute run is made in which the flotation pressure is accurately maintained and plotted every minute. A major disturbance is now introduced by putting 10 centimeters Hg pressure on the diver until within a minute the bubble has almost disappeared by dissolving into the water. The diver is again brought to flotation, and faster than it can be plotted, it regains its former flotation pressure within a half millimeter Hg. In other words, even major disturbances, involving the new generation of the gas bubble from the dissolved gas, are repaired within 30 seconds. Clearly, therefore, we can accurately record even very rapid respiratory changes with very little damping effect (Linderstrøm-Lang, 1946).

D. *Sensitivity*

The sensitivity of the system depends upon several factors: (a) on the smallest increment or decrement of the bubble volume that will move the diver up or down, (b) on the magnitude of the pressure change necessary to produce this volume change, and (c) on the total pressure.

a. The method is essentially a "gravimetric" method, and therefore the greatest sensitivity will be obtained when the forces opposing the buoyancy changes are smallest. These involve the masses to be accelerated and the frictional forces. By using a reference diver instead of floating the whole respiratory chamber it is possible at once to reduce very drastically both the mass and the friction. The mass of the reference diver corresponds to around 150–200 $\mu\mu$l. The micro Cartesian diver has a moving mass, when floating in the medium of specific gravity 1.3, of about 2.5 mg., *i.e.*, some 12,000 times more mass to accelerate than the reference diver. The viscosity of fresh (and sea) water for floating the reference diver is only half that of the flotation medium used for Cartesian divers.

b. The pressure necessary to convey a volume change to the diver gas depends upon the number of menisci to be moved or deformed. The reference diver has one meniscus, whereas the micro diver involves four. The present size reference diver can be held within about 0.2 millimeter Hg, and we have not so far tried a water manometer. During oxygen consumption, when the flotation pressure is steadily decreasing, the accuracy of defining the flotation pressure undoubtedly goes down, somewhat in proportion to the rate of pressure change.

c. The sensitivity bears a direct relation to the total pressure at which the diver is operated. This is apparent if we consider the least molar amount of gas added or withdrawn that we can measure. The resulting volume change will depend upon the ratio of the amount withdrawn to the total amount present in the system. The sensitivity related to molar amounts of gas, therefore, increases in an inverse proportion to the pressure. The higher sensitivity at lower pressures is clearly felt in the manipulation of the syringe.

E. *Stability in time*

The stability of the flotation pressure is measured by blank runs. It depends on several factors, the most important ones being: (1) constancy of the temperature of the water bath and of the manometer, and (2) diffusion of gas into the chamber from the water trapped in the capillary space between the rod and the chamber. Other sources which, especially during respiratory runs, could cause a shift of the base line would be (3) changes in surface tension, (4) changes in water vapor tension and density of the medium, (5) changes in area of bubble attachment to the diver, with consequent change in diameter and pressure.

On Figure 7 are given curves of the stability of the flotation pressure as found in several series of blank runs in fresh water, sea water and some media. The stability of the flotation pressure seems largely independent of the chamber size. The stability in absolute volume units can therefore be taken as nearly proportional to the chamber size, *i.e.*, the factor F (p. 164). On the chart the factors actually went from 0.7 to 10, *i.e.*, one mm. Hg corresponded to from 0.7 to 10 $\mu\mu$l.

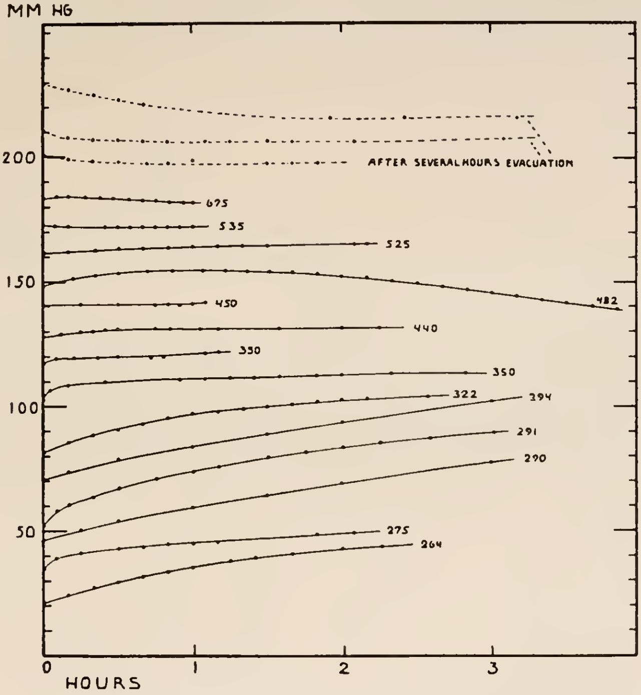

FIGURE 7. Stability of flotation pressure in blank runs. The number at the end of each curve plus 70 gives the flotation pressure in mm. Hg at the beginning of the blank run.

1. *Effect of temperature changes*

From equation 1 we may calculate the temperature sensitivity of the system by knowing the amount by which the gas bubble, the solubility coefficient, the water vapor tension and the surface tension change with temperature. If we move from 20° to 21° C., α_{O_2} changes from 0.03102 to 0.03044, α_{CO_2} from 0.878 to 0.854, water vapor tension from 17.54 to 18.65 mm. Hg, and bubble pressure due to surface tension from 31.18 to 31.11 mm. Hg. If the bubble volume is

180 $\mu\mu$l and the liquid volume is 20000 $\mu\mu$l and the gas (O_2) is at 760 mm., this rise in temperature will change the pressure by 13.3 mm. Hg. A hundredth of a degree will change the pressure near to 0.13 mm. Hg.

It may similarly be calculated that when all the O_2 of the system (802 $\mu\mu$l) has been used up and 802 $\mu\mu$l CO_2 have been produced, the resultant total pressure will be 20.8 mm. Hg at 20°. A one degree rise will then produce a 2.1 mm. increase in pressure, and 0.01° therefore 0.02 mm. increase.

If the system were all a moist gas phase it can similarly be shown that the same rise in temperature would raise the flotation pressure only 4 mm. We see thus that the temperature sensitivity increases: (1) the more liquid there is compared to gas, and (2) the higher the total gas tension of the system is.

The reason for the high sensitivity of a high liquid ratio lies in the fact that the all gas phase changes as $\frac{T}{T_1} = \frac{294}{293}$, whereas the all liquid phase tension changes as the ratio of the solubility coefficients $= \frac{0.03102}{0.03044}$, which is a 5.6 times greater change. Similarly the pressure change of CO_2 in an all liquid system is 8.2 times as temperature sensitive as in an all gas phase, when the initial pressure is the same. In other words, the temperature effect on the solubility coefficients is much greater than on the gases themselves.

A compensating chamber is very commonly used in gasometric methods in order to cut down the temperature sensitivity of the system. It should be borne in mind, however, that a large stationary compensating chamber will not compensate for the temperature effect on the solubility coefficients of the gases. If the liquid-to-gas ratio in the respiration chamber is 10/1 or 2/1, a temperature change from 20° to 21° will leave the system uncompensated by, respectively, 40 and 10 mm. water. In such extremely sensitive systems as the Cartesian diver, it is therefore essential to keep an exceptionally accurate temperature control, especially so where the liquid-to-gas ratio is high.

We may conclude from the above calculations, which have been verified also by experiments, that we need to keep the water bath constant to within 0.01 degree to obtain stability of the flotation pressure within 0.2 mm. Hg. If we were to use a water manometer to be read with the same linear accuracy we would have to keep the temperature to within 0.001 degree.

It can easily be calculated that the mercury barometer must be kept within 2° C. in order to match the temperature sensitivity of the water bath, *i.e.*, ±0.01°.

2. *Diffusion of gases from the sleeve*

The blank curves (Fig. 7) are arranged in a sequence, downwards according to the initial pressure reading, which is given in mm. Hg at the end of each curve. It will be seen that the lower curves, with a low flotation pressure, all show an initial rise, which in the first hour may amount to as much as 10 mm. Hg. This increase in flotation pressure diminishes, or disappears, as the flotation pressure approaches atmospheric pressure. The rise is due to gas diffusing in from the water trapped at one atmosphere's pressure in the capillary sleeve between the rod and the chamber wall. This can be proved by evacuating the loaded chamber for several hours, so that finally the sleeve water gets a low gas tension. When, now, the flotation pressure is established, gas will diffuse back into the sleeve water from the chamber, producing a slight decrease in the flotation pressure (see the three upper curves).

We have not been able to demonstrate any measurable diffusion of gas *through* the sleeve. Even such drastic measures as changing the outside water from vacuum extracted to fully saturated water leave the flotation pressure undisturbed. Evidently diffusion through the capillary sleeve is exceedingly slow.

The pumping action produced by the slight volume changes in the bubble while adjusting the flotation pressure is much too small to effect any water renewal in the sleeve. It has so far been impossible even with gross changes in the bubble volume by vacuum to pump any dyed outside liquid into the chamber.

As the pressure during a run decreases the sleeve presumably continues to yield a small amount of gas proportional to the decrease in pressure. This will make a slight error in the chamber factor. Considering that the chamber factor and the cell volume are not very exactly known, the sleeve error is unimportant.

3. *Effect of changes in surface tension*

Any change of the surface tension of the diver bubble would change the bubble pressure, and this change would be reflected as such on the flotation pressure. During an experiment the medium is continuously changing with respect to gas tensions, pH, and organic and inorganic solutes due to the presence of the organism. Ferguson (1914) has shown that one atmosphere of CO_2 gas has very little effect on the surface tension of water, so that the maximum effect from CO_2 in our system would be entirely negligible. A negligible effect would also be produced by relevant changes in the concentrations of NaCl, KCl, urea, glycerine, sucrose, sodium phosphate, ammonium chloride (Landolt-Börnstein, 1923; McClendon, 1917). High molecular constituents of urine, bile and plasma may, however, significantly lower the surface tension. It would be of no significance for the method if a medium had a different surface tension from that of water, as long as it remained constant. It would not matter, either, if it showed a slow linear change, as this would only affect the factor converting pressure to absolute units of O_2 consumption. A sudden release of a surface acting substance (*e.g.*, protein) could give a sudden parallel displacement of the rate curve, but would then leave the rate unchanged. As surface acting substances are concentrated in the surface, they tend to exercise near their full action already at minute concentrations. If such substances were released periodically, it seems therefore likely that they would lead to only small periodic changes in the surface tension.

4. *Changes in water vapor tension and density of medium*

Only a change in the water vapor tension during the experiment will affect the results. A gradual change would be of no consequence, as both the absolute rate and the amount of respiring material would be less well known. Measurable sudden changes in water tension are even more unlikely than sudden changes in surface tension, and would tend to be counteracted by the density change.

5. *Changes in bubble diameter*

There is a possibility of a pressure change in the bubble if it changes its adhesion surface to the diver. This could happen if it does not fully occupy the whole cut surface.

The salt water diver has an oblique upper surface with no boundaries set for the bubble, which therefore could be assumed to vary in its attachment surface, and hence radius and pressure. In the experiments referred to on page 168, where the diver was subjected to gross transitory pressure changes, no changes in the final flotation pressures were detected, and hence there were no measurable changes in bubble radius. For tension determinations where it is necessary to generate the bubble twice, the diver is so long that the bubble will always completely occupy the cut surface.

III. Procedure

1. Preparation of sterile material and chamber

It seems that bacterial contamination is a more conspicuous menace in this method than in macro methods. It is essential always to keep this in mind and try to avoid insidious errors from this source. Nutritive media especially, such as, for instance, hay tea or even sea water, must be very carefully sterilized and handled. Sea water was filtered through a porcelain candle (Selas, porosity 015) and then heated overnight in a thermostat of 70° C., allowed to stand at room temperature the next day, and then heated again to 70° C. The cool water was shaken with air and poured into two or three covered sterile Syracuse dishes.

All instruments and glass tubes, tips, vials, dishes and respiration chambers with rods must be boiled or autoclaved immediately before use.

The cell is transferred by means of a braking pipette into the water or medium of one dish, where it is allowed to sink or swim about. The pipette is rinsed several times in sterile water and the cell similarly transferred to two more dishes.

2. Loading the chamber (Fig. 8)

The medium is poured into a tube which is placed in a beaker of ice water and bubbled with moistened oxygen. The tube is kept loosely covered. After half an hour there will be enough oxygen in the water. The respiration chamber is "threaded" up on the suction tip, where it is held by the suction. The tube with bubbler is lifted up submerging the respiratory chamber, which thereby is filled and rinsed. The chamber is gripped by forceps while completely submerged, and the whole assembly is lowered as one piece down from the suction tip, leaving the respirometer chamber filled. The chamber is removed from the liquid and rolled up under a hook on the rubber stopper that serves as a chamber holder. It is imperative that there be no air bubbles sticking to the walls of the chamber. This is ascertained under the dissecting microscope. The cell is now transferred to a dish where it is allowed to sink through a centimeter or so of sterile medium and is then transferred into the opening of the chamber with a fine braking pipette. If the cell readily sinks, the chamber holder is put on its flat end. If the cell swims or does not freely sink to the closed end of the chamber, the chamber is placed in a simple cork centrifuge which is run very gently from a Variac. The chamber is then transferred back to the holder under the binocular.

At this point the diver is cut as described (p. 161), or picked up with a braking pipette from its storage drop.[9] While still on the knife tip it is transferred to the meniscus of the chamber where it will adhere to the surface. Using a fine needle, it is pushed into the liquid, unless deposited by a pipette. The stopper is held with chamber opening up and a slight tapping is usually enough to bring the diver down to the bottom next to the cell.

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[9] The diver filament will not stand heat sterilization. It may be wiped off with alcohol and water, or only water.

The rod is now rinsed off in the same medium from which the cell was taken. One of the drops adhering to it is touched to the chamber opening so the meniscus is convex and the solid rod is now inserted. In case the cell is a swimming organism like Paramecium, it may be quite a trick to get the rod in, using a combination of centrifugation and slight advances every time the animal moves in until the rod is finally seated. It is possible to some extent to feel how the rod should be rotated to seat deepest. Any quick pull back must be avoided, as this cavitates a bubble onto the diver and a positive flotation pressure may result.

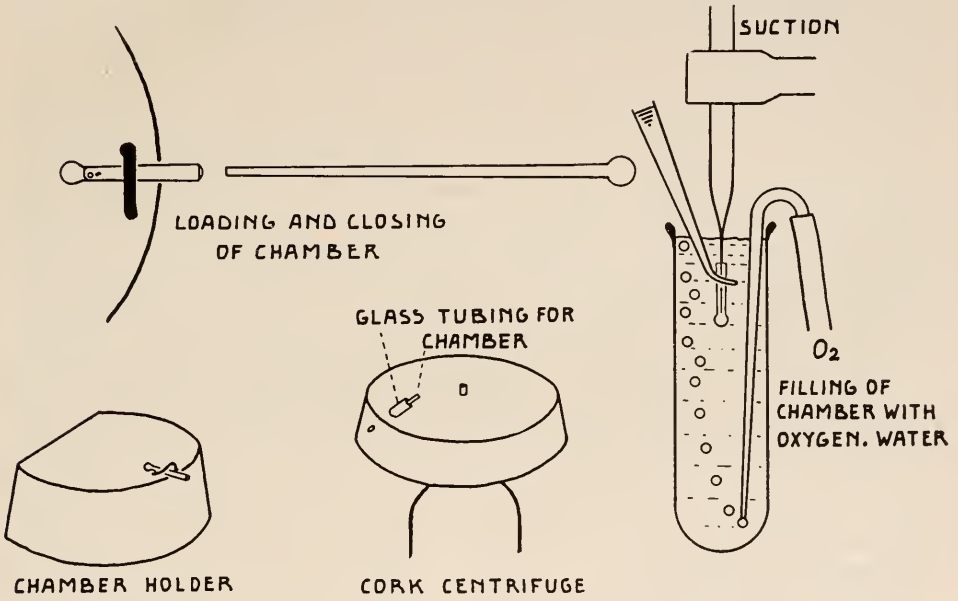
SUCTION
LOADING AND CLOSING OF CHAMBER
GLASS TUBING FOR CHAMBER
O_2
FILLING OF CHAMBER WITH OXYGEN. WATER
CHAMBER HOLDER
CORK CENTRIFUGE

FIGURE 8. Operations involved in loading the chamber with oxygenated medium, cell and diver.

The chamber, rod down, is now put into the chamber container, which is filled two thirds with the medium. The bubble fiber (p. 160) is put in and the container clamped in place on the water bath.

3. *Measurement of chamber*

At this time it is convenient to measure the chamber height and diameter by the ocular micrometer, and also the height of the diver from the base to the opening of the capillary hole. If a measurable cell is being used, like an egg cell, its diameter is also measured. It is often possible at once to calculate the chamber volume from the dimensions by using the chart mentioned on page 164, and from another chart (p. 175) read off the factor which translates mm. Hg to $\mu\mu$l O_2.

Especially if working with an assistant, one can then usually have the readings calculated and plotted during the run.

4. *Floating the reference diver* (*Fig. 3*)

For this step to succeed, it is necessary that the chamber and the rod be absolutely clean so the diver will not stick. With fresh water the walls are usually hydrophilic, a straight cut diver can be used and the flotation is easily accomplished. In sea water the walls become hydrophobic and flotation cannot be accomplished unless the bubble is kept from touching the glass walls. There are two precautions taken to make flotation possible: (1) shape of the diver; (2) orientation of the diver before generating the bubble.

The diver is shaped so that when it sinks and hits the bottom it tends to fall with the bubble surface upwards, and when it floats the bubble cannot touch the sides of the chamber.

To orient the diver the tube is rotated and tapped and tilted until the diver lands right side up on the bottom with the head end away from the wall and free from the cell. The tube is now turned so that the diver is seen in side view; it is connected to the manometer system and a slight vacuum admitted, enough to suck in the plunger of the syringe. The connection to the manometer is clamped. The vacuum is carefully increased by turning the syringe knob until the gas meniscus in the capillary of the diver moves out making a bubble. This will rapidly expand unless checked by the syringe. While lightly tapping the tube with a fine glass rod, the bubble is expanded until the diver rises and finally floats free from the bottom. It must not be allowed to hit the ceiling. When the pressure is stable, the clamp is barely opened and immediately closed, causing the diver to rise or sink, this being immediately counteracted by the syringe. If the diver sinks, the manometer pressure is lowered a few centimeters by the stopcock and the flotation pressure is again checked until the manometer pressure matches the flotation pressure and the clamp can be taken off. If the diver floats too near a wall, it can be brought near the middle of the chamber by tilting the chamber towards horizontal, bringing the diver up a little and then down when the chamber is again vertical.

If during these manipulations the diver gets stuck with the bubble against the bottom, top or side walls, pressure is put on to near atmospheric. The syringe plunger is pulled half way out and the clamp tightened. By applying overpressure with the syringe the bubble is made to disappear into the diver capillary and the diver is again free and can be re-oriented before a new flotation is attempted. If the diver itself sticks, it can usually be shaken loose if the chamber tube is removed from the water bath and given a violent shake down.

If the bubble inadvertently should dissolve completely, a new one usually cannot be formed by simple evacuation. There is still one chance left to save the run, namely, by cavitating a bubble onto the diver. To do this the chamber is removed from the tube and is held submerged in a dish of the medium. The rod is barely loosened very carefully and immediately pushed back in. This cavitates a bubble onto the diver which then often can be made to float. It is immaterial where the bubble forms on the diver. The flotation pressure is often high as a result of this procedure, sometimes even higher than atmospheric pressure. In such case air must be blown into the barometer flask and the syringe must be manipulated with two hands, one continually pressing the barrel in, while the other regulates the motion.

5. *Recording of the flotation pressure*

With the reference diver afloat, the measurements can start, and readings can be taken at any suitable interval. The ocular scale is oriented in a vertical position, and the middle line of it is placed a little above the middle of the chamber. The distance between this line and the bottom of the chamber is maintained throughout the run, and the bubble is held so that the top of it touches the ocular line.

For most accurate readings, especially if close time intervals are desired, it is best to have an assistant start calling off every five seconds a half minute before reading time. During this period the diver is held at the mark very accurately and excursions of the pressure regulation are made as smooth and as small as possible. It is wise to check the water bath temperature at every reading and note it down.

6. *Re-setting of manometer pressure*

The syringe serves as fine regulation and its capacity is soon exhausted, especially when the total pressure is low. To re-set the pressure the stop-cock on the barometer flask is rapidly turned past the vacuum connection. This lowers the pressure by a few centimeters, and the diver rises. It is immediately brought back to the line by letting the plunger in. The procedure is repeated until the plunger is far enough in to last for some time.

7. *Removing cell and diver from the chamber*

It is sometimes useful to be able to remove the cell and diver from the chamber, so that the same run can be repeated later or with a different medium, or it may be desirable to measure the cell volume or other properties of the cell that was used. In order to do this, the chamber is taken out of the tube and is held in a Petri dish containing the medium. With the sleeve covered by the liquid the rod is very gently pulled out, filling the chamber with the medium. The chamber is then placed, opening down, in a small dish of the medium, letting the diver and the cell fall out.

8. *Calculation of results*

It is customary in micro respirometry to recalculate the readings as increasing from the first one, which is called zero, by subtracting all the subsequent readings from the first. In a detailed study all these figures, showing the change in pressure as a function of time, must be recalculated to give $\mu\mu$l O_2 consumed. This is done by first calculating the liquid volume as described on page 164 and then using the formula (8). If the diameter of the diver filament is kept constant and the diver length reasonably constant, a great deal of calculation can be saved by computing the factor for four situations, using two different chamber volumes, of, say, 20,000 $\mu\mu$l and 100,000 $\mu\mu$l, and two different diver lengths, one with height of the diver equal to the diameter and the other equal to one and a half times the diameter. A graph is prepared with the factors on the ordinate and V_L on the abscissa. The four points are plotted and the pairs connected with parallel lines. In the relevant region the function (equation 8) is very nearly a straight line, and the factor is found by interpolation.

This chart can of course only be used in series where the temperature, salinity and diver diameter are kept the same.

We are greatly indebted to the officers of the William F. Milton Fund for making this development possible.

We wish to express our appreciation to Dr. A. Baird Hastings for constant encouragement and advice. He has forcefully backed the project from its first vague beginning. Miss Barbara Orski has contributed several blank runs, and Mrs. Susan I. Scholander has given much technical and secretarial help during the later stages of the development. We wish to convey to these ladies our affectionate thanks.

SUMMARY

1. A method for micro respiratory studies is described with a sensitivity down to 0.2 millionth of a cubic millimeter and a stability of 10–0 millionths of a cubic millimeter an hour. It is basically a constant volume method, where a minute reference diver is introduced into the chamber together with the cell. A bubble is generated on the diver until it just floats. The change in flotation pressure multiplied by a factor of known constants gives the oxygen consumption. The CO_2 is effectively held in solution by the high liquid-to-gas ratio. The system has been used to study the respiration of single protozoans and to follow in detail the oxygen consumption during cleavage of single eggs of several species, which will be reported elsewhere.

2. A new micro method is described for direct determinations of gas tensions in liquids using the reference diver.

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RESPIRATORY STUDIES OF SINGLE CELLS. II. OBSERVATIONS ON THE OXYGEN CONSUMPTION IN SINGLE PROTOZOANS [1]

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During the development of the reference diver technique (Scholander, Claff and Sveinsson, 1952) for studying single cell respiration, many measurements were made on single protozoans. We are here presenting observations on (1) the relation between cell volume and respiratory rate as determined over a thousandfold range in size, using individual cells from three species of protozoans, and (2) the metabolic change occurring at excystment and encystment of *Bresslaua.*

Material and Methods

Respiratory measurements

A single animal was pipetted from the stock culture, usually hay tea with a few wheat grains, and transferred with a minimum amount of water into a dish containing sterile Ringer solution diluted 50 times. Two to three additional transfers were made, where the cell was allowed to swim through, or was moved through, relatively enormous amounts of sterile solution. If any bacteria were left clinging to the cell the inorganic medium would hardly support any appreciable growth during the time of the experiment. All measurements were made at 25° C. and each run lasted 60–70 minutes. Our runs confirm the findings by Amberson (1928) that the oxygen rate stays constant in spite of large changes in the oxygen tension. After the run the cell was transferred to a dish and the cell volume could subsequently be measured.

Measurement of cell volume (Fig. 1, A and B)

The cell volumes were measured by squeezing the cells between two glass surfaces spaced at a known distance, and tracing the surface contour. Mast and Fowler (1935) and Pèterfi and Maleci (1938) used a similar method of deforming the cells, by sucking them into a capillary tubing of known diameter and measuring the length.

For the measurements two brass wire frames 1 × 2 cm. are prepared. Stretched across the frame on the under side are two platinum filaments [2] of accurately known diameter. One frame had filaments of 19 μ, the other of 38 μ. A 10 × 5 mm. piece of a microscope slide serves as a thick and heavy coverslip. The frame is placed on a microscope slide, and the cell in a small drop of water is placed between the filaments. The cover glass is carefully put on. Dust must be carefully avoided on the spacer filaments. A paramecium can move about slowly even when squeezed flat to about one fifth or one sixth of its normal diameter. When at rest its circumference is rapidly traced through an Abbé prism onto a piece of paper which has a known weight per surface area. The cell tracing is cut out and weighed and the cell volume is calculated from the surface area and the thickness. In one species of *Paramecium* several measurements on the same individuals gave an agreement within ± 6%.

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[1] This work was supported by a grant from the William F. Milton Fund.

[2] Obtained from Baker and Co., Newark, N. J.

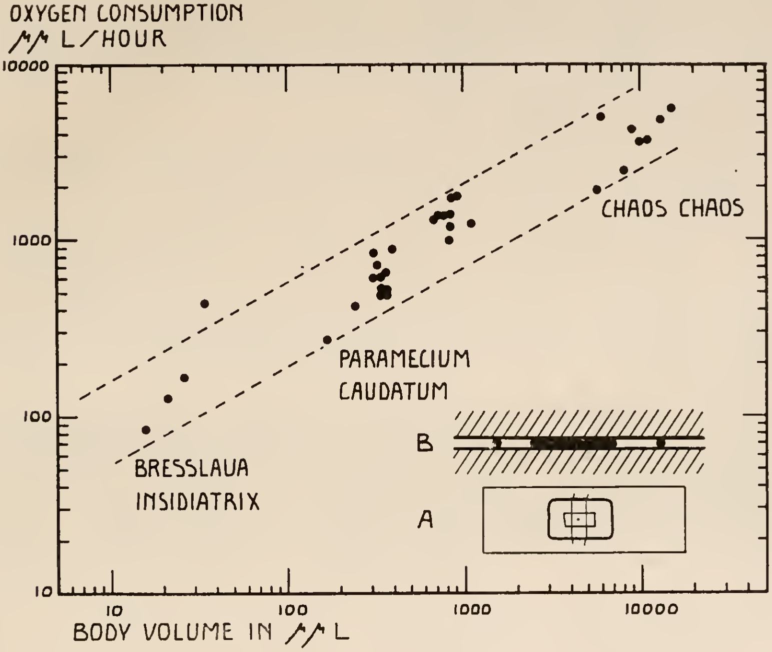

FIGURE 1. Ratio of oxygen consumption to cell volume, both determined in the same individual cell. The parallel lines indicate the general trend of the data. The slope of the lines is the exponent n of the equation $M = K \times W^n$, where M is the oxygen consumption, K is a proportionality constant and W is the weight. We attach no significance to the empirical value of n fitting these data. Insert A. Method for measuring cell volume by squeezing cell to a known thickness under a cover glass resting on two spacing filaments. Insert B. Cross section showing spacing filaments and flattened cell between the glass surfaces. One $\mu\mu$l = one millionth of one mm^3.

The amoeba *Chaos chaos*, which cannot be flattened nearly as much as a paramecium without rupturing, was squeezed between thicker filaments or even under the coverslip of a blood cell counting chamber. In order to succeed in the measurement it is necessary to tease the amoeba to contract by tapping the glass and trace it quickly before the protoplasm begins to flow out at the edges. In the amoeba the volume measurements are probably not better than ± 10%.

RELATION OF SIZE TO OXYGEN CONSUMPTION IN SOME PROTOZOANS

Using the reference diver technique we have determined the oxygen consumption in a series of cells of different species and size. The absolute accuracies obtained are estimated to be within about ± 10% for the smaller species and ± 5% for the larger species.

There seem to be very few cases where metabolic rate and cell volume are given for the same cell. Holter and Zeuthen (1948) give such data for amoebae (*Chaos chaos*), with the respiration measured by the Cartesian diver technique, and the cell volume determined colorimetrically (Holter, 1945), or estimated from the buoyancy of the cell in water (Zeuthen, 1948). In another amoeba, *Difflugia* sp., the oxygen consumption was determined individually in seven animals and the average size is given (Zeuthen, 1943).

There are many observations available where the average oxygen consumption per individual can be calculated from estimation of the number of cells used in mass runs, and where the average cell volume can be estimated from linear measurements or a hematocrit technique. These runs have often yielded extremely variable results. The figures given for one of the most studied protozoans, *Paramecium caudatum,* range from 120 $\mu\mu$l/hour/cell to 5600 $\mu\mu$l (*cp.* compilations by von Brand, 1935, and Jahn, 1941). These very large discrepancies are sometimes due to inadequate methods. In many cases bacterial infection may have been a large source of error.

Especially in mammals it has been well established that the resting metabolic rate (M) can be described as proportional to an exponential function of the weight (W), according to $M = K \times W^n$. The exponent n has been found empirically to be near 0.75 (see Brody, 1945; Kleiber, 1947). There are many known exceptions to this rule (see Scholander, Hock, Walters and Irving, 1950). A great deal of similar information has also been collected for poikilotherms (see Zeuthen, 1947; Hemmingsen, 1950). When the metabolic rate and the weight are presented on a double log plot the exponent will appear as the slope of the line of correlation.

In Figure 1 we have accordingly plotted the oxygen consumption versus the volume of the cell, and in this material of a thousandfold range in size, the exponent (slope) is low, about 0.55. If the dotted parallel lines are extended down to the size of bacteria, we will hit *Bacterium coli* at a cell volume of 0.5–1 μ^3 and an O_2 consumption of 0.1–1 $\mu\mu$l/cell/hour (Martin, 1932; Huntington and Winslow, 1937; Hershey and Bronfenbrenner, 1938), but we will be about ten times too high for *Photobacterium phosphorescens,* which, with a volume of 1.7 μ^3, has only 0.06 $\mu\mu$l O_2 consumption (Harvey, 1928). If we start at the upper end, the data on *Chaos chaos* by Holter and Zeuthen (1948) fit well between the lines and so do the data on *Difflugia* when the shell is disregarded (Zeuthen, 1943). The oxygen consumption of *Tetrahymena geleii,* with 15–24 $\mu\mu$l cell volume, has been found by several investigators to lie between the lines (see Ormsbee, 1942; Pace and Lyman, 1947), but it may rise up to three to four times higher than the upper line when given suitable substrate. *Astasia klebsii,* on the other hand (Pringsheim, 1936; von Dach, 1940, 1942), was found about twenty times lower than the "*coli-chaos*" line when starved, but on the line when fed on acetate (cell volume about 5 $\mu\mu$l, O_2 consumption 2–4 $\mu\mu$l/hour). *Sarcina,* with a cell volume of near 2 μ^3, was found by

Gerard and Falk (1931) to use about 0.007 $\mu\mu$l/cell/hour, which places it about 100 times below the lines.

It seems that these samples of data from careful investigations point towards a very considerable variation in the relation between size and metabolic rate in the range of single cell organisms. It would appear impossible to fit the data into any simple rule, such as the "mouse to elephant" curve (Benedict, 1938), or even the "beech tree to egg of silkworm" curve discussed by Hemmingsen (1950). When interpreting the available data on single cells this author arrives at an exponent near 1, whereas according to our data and compilation many forms would fit a much lower exponent. The difficulty undoubtedly lies in the fact that we can only very poorly define what we are correlating. Although on a volume or weight basis a large number of more or less "standard" cold-blooded organisms do fit a certain exponential line, it cannot be expected that the same relation will hold also for forms that carry vast amounts of metabolically inactive material, such as corals, jellyfish, eggs, trees, etc., unless possibly some correction for the inert material could be made. In many forms (seeds, spores, etc.) it is likewise very difficult to define what is meant by resting rate. These difficulties are certainly no less in dealing with the single cell organisms.

In our series, we are comparing fast swimming, highly active *Paramecium* and *Bresslaua* with extremely sluggish amoebae, and we would not expect that both would belong on the same line unless the active ones were resting. In our material we have not seen any striking changes in O_2 consumption due to locomotion in the *Paramecium,* but we have not as yet studied it extensively, and we do not believe that the 200–300% spread in the data can be explained on this basis. To what extent locomotion or other activity influences the oxygen consumption in protozoans can undoubtedly be studied by the reference diver technique. The lines drawn in Figure 1 are purely descriptive for the material presented, and are at the present time void of any other significance.

Excystment and Encystment in Bresslaua

Bresslaua insidiatrix (Claff, Dewey and Kidder, 1941) will form a protective resting cyst if left long enough in pure water. It will start to circle and to eject a protective cyst shell in which it keeps turning for several hours until it finally stops. It can stay dried up as an undifferentiated, spherical, resting cyst for years. If this cyst is placed in hay tea it will start to differentiate and will excyst within an hour or so. A pulsating vacuole is formed, the animal starts circling inside the cyst shell and finally breaks through.

Gregg (1947) measured the effect of encystment on this organism, using three individuals simultaneously in a micro modification of the Warburg respirometer. He observed a 50% decrease in the oxygen consumption when the cells encysted. The rate of oxygen consumption of the free swimming cell was found to be about 10,000 $\mu\mu$l/cell/hour at a cell volume which, estimated from the linear dimensions given, cannot have been more than 500 $\mu\mu$l. This gives about ten times higher rate than indicated by the present method.

The respiratory events at excystment and encystment were followed in the respirometer, using single cysts or cells (Fig. 2). The O_2 consumption in resting cysts was immeasurable by this method. In order to effect excystment the cyst was placed for five minutes in sterile hay tea and then transferred several times in sterile 1/50 dilute Ringer solution in which it was run. Hay tea is a better medium for excystment but was found too difficult to maintain sterile.

After the activation by hay tea there is a steady increase in oxygen consumption, and at the moment the organism breaks through the cyst there is a definite but often quite small rise. It is conceivable that this rise is merely due to the elimination of the shell, which would act as a limiting factor for the oxygen diffusion at the high metabolic rate of the fully developed cell.

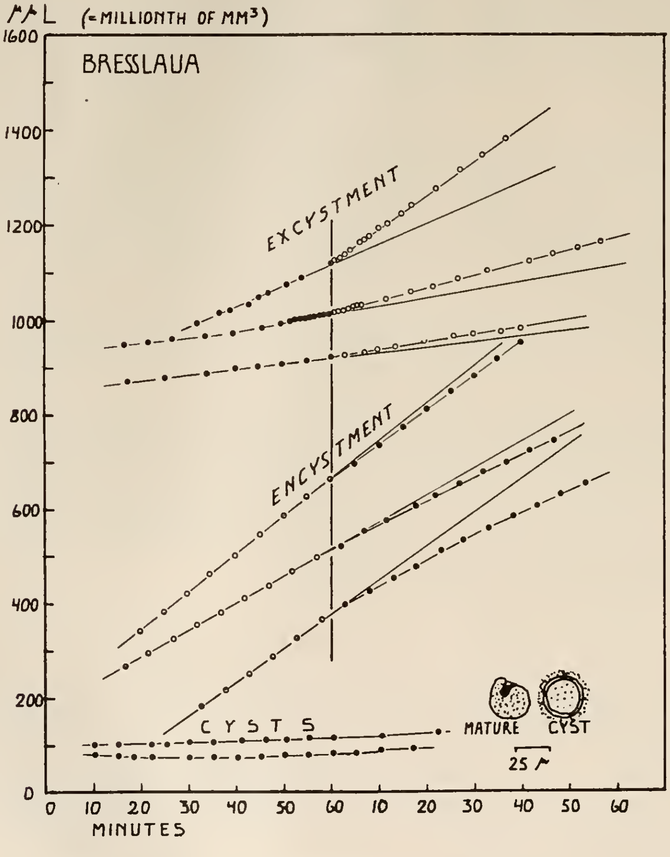

FIGURE 2. Oxygen consumption at excystment and encystment in single individuals of *Bresslaua insidiatrix.*

In Figure 2 three runs are given showing the reverse effect on the oxygen consumption when the cells are encysting.

We wish to express our gratitude to Dr. A. Baird Hastings for much helpful advice during the work and to the officers of the William F. Milton Fund for supporting this investigation.

Summary

1. Observations are reported on the oxygen consumption of single individuals of four species of protozoan using the reference diver technique.

2. The relation of oxygen consumption to cell volume is given for a series of individual protozoans covering a thousandfold range in size, and the change in oxygen consumption following excystment and encystment in *Bresslaua* has been recorded.

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RESPIRATORY STUDIES OF SINGLE CELLS.[1] III. OXYGEN CONSUMPTION DURING CELL DIVISION

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The reference diver technique (Scholander, Claff and Sveinsson, 1952a) has made it possible to study in detail the oxygen consumption of large single cells. In a previous paper (Scholander, Claff and Sveinsson, 1952b) we reported observations on single protozoans, and in the present study we present data on the oxygen consumption during the first four cell divisions of the fertilized eggs of two sea urchins, *Strongylocentrotus purpuratus* and *S. franciscanus*, a sand dollar, *Dendraster excentricus,* and the echiuroid worm, *Urechis caupo.*

During mitosis a series of events takes place, all of which require energy in one form or another. These events could conceivably occur within the limits of a perfectly steady total flow of energy expenditure or they might present themselves as bumps or cycles on top of a "basal" energy exchange. The gross process of cleaving one lump of protoplasm into two or more pieces is one phase which could readily be expected to be associated with such an excess oxygen intake. In aerobic eggs the energy necessary for mitosis may in the last analysis be oxidative, and it is therefore natural to look for cycles in the oxygen consumption correlated to the anatomical events. Many authors have looked for such cycles in the oxygen consumption with both positive and negative results. For a complete survey the reader is referred to the excellent monographs by Needham (1931) and Brachet (1950).

Gray (1925) in two mass run measurements on *Echinus esculentus,* using a Barcroft respirometer, found no cycling in the oxygen consumption. By re-calculating and plotting his data according to the procedure which is described below, it is apparent that in one of his runs there are actually very slight accelerations in the rate associated with each of the three cleavages.

Runnström (1933a), using the Warburg apparatus, in mass runs with *Paracentrotus lividus*, found an increase in oxygen consumption starting at the appearance of the cleavage furrow. Brachet (1932, 1934, 1935, 1950), using in most experiments a Fenn respirometer, found cycling in the oxygen consumption of frog eggs (*Rana*

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[1] This work was supported by a grant from the William F. Milton Fund.

fusca). In 34 runs with some 80 eggs per respirometer, he found that the peaks of his curves coincided with the appearances of the cleavage furrow.

Zeuthen (1949, 1950a, 1950b, 1950c) found cycling to be of constant occurrence in eggs of *Psammechinus miliaris, Strongylocentrotus franciscanus, Dendraster excentricus* and *Urechis caupo,* which were run several hundred at a time in modified Cartesian divers. The cleavage usually took place when the rate of oxygen consumption was at a minimum. In all of the echinoderms mentioned he found that the cycling increased markedly in intensity after the fifth to seventh division. Tang (1948) reports similar results on *Arbacia punctulata,* using the Warburg technique.

In all mass runs one must depend upon the degree of synchronism of the cleavages of a great number of eggs. There is necessarily some individual variability in the cleavage rhythm, and one faces the risk of promoting asynchronism by tension gradients developing in a sedimented cell mass. Whatever the reasons may be for differences in the individual rhythm of eggs, it would seem likely that the cell divisions would get progressively more out of step at each successive division. As amply demonstrated, especially by Zeuthen, the statistical approach has yielded extremely valuable information. A certain degree of blurring of the details is inevitable in mass determinations, however, and it is hence of interest whenever possible to supplement the information by runs on single eggs. This has hitherto been possible only for very large eggs, such as that of the frog. Zeuthen (1946) studied single frog eggs (*Rana platyrrhina*) in modified Cartesian divers, and found that cycling was a regular feature, with cleavage beginning at or slightly after a respiratory minimum. Cycling was also found to take place without cytoplasmic division.

In the present investigation we have undertaken to analyze on single eggs the oxygen consumption during cell division in several marine species. With the exception of *S. purpuratus,* Zeuthen (1949, 1950c) has studied the same forms in mass runs.

Material and Methods

The echinoderm material was secured at Friday Harbor, Washington, and *Urechis* at Pacific Grove, California.

Eggs and sperm from *S. purpuratus* and *Dendraster* were obtained either by spontaneous spawning or by injection of isotonic KCl solution. The spawning season was over for *S. franciscanus,* but enough eggs for our purpose were obtained by removing the ovary and shaking pieces of it in cold sea water, washing out a dozen eggs or more. The eggs were then rinsed free of all debris in several dishes of sterile and cool sea water. All dishes were kept cool.

Dendraster has a thick and colorful "furry" coating on the egg. This was stripped off from the fertilization membrane by passing the egg repeatedly through a narrow pipette opening.

The eggs and sperm of *Urechis* were taken by pipette from the genital pores. In this species all runs were performed on material from the same two animals, whereas in the echinoderms new pairs were used each time. Much difficulty was encountered with the *Urechis* eggs in the beginning because the egg adhered badly to the chamber wall and made the diver stick. It required many passings of the egg through a pipette to get rid of this stickiness.

At zero time the eggs were fertilized by stirring them with a fine glass tip which had been touched to a cold and concentrated sperm suspension. The fertilization was followed under a dissecting microscope and as soon as the membrane had developed, a perfect-looking egg was picked for the run. This egg was transferred through two or three dishes of cold sterile sea water and loaded into the chamber, which was kept cold by having the supporting stopper chilled beforehand. The centrifuge was kept in the refrigerator. Sterile technique was used throughout (see Scholander *et al.*, 1952a).

Presentation of Data

In Figure 1 the cumulative oxygen consumption of the *Dendraster* runs has been plotted in $\mu\mu$l, omitting most readings that are less than three minutes apart. Under each of these curves we have drawn a straight line representing an average rate measured in $\mu\mu$l/min. We are particularly interested in seeing to what degree the observed curves deviate from the straight lines. To find this we calculated a series of figures for the average oxygen consumption corresponding in time to the observed

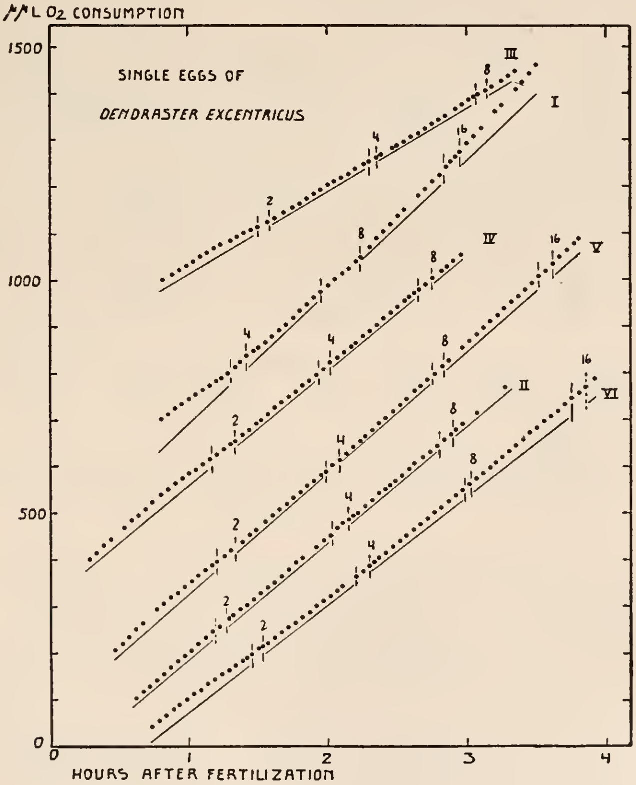

FIGURE 1. Cumulative total oxygen consumption during cell divisions in single eggs of the sand dollar, *Dendraster excentricus*. The beginning and end of each cell division are marked by vertical lines and the number of cells at the end of the division is indicated. Under each curve is drawn a straight line taken as the average rate of consumption. The position of each curve on the ordinate scale is arbitrary.

figures. The two sets of figures were subtracted from each other point by point, giving the absolute deviations from linearity in μμl. The deviations were plotted at their proper times and could now be sufficiently enlarged to permit a detailed analysis. The curve thus obtained is slightly skewed to the left because the values are plotted at an angle from the original ordinates. This effect is entirely negligible when the oscillations are as small as those presented. We have chosen this way of treating the data rather than recalculating the observations as a change of rate from point to point. The latter procedure has been most commonly used but is wasteful of good data if the observation points are very close together. The present curves represent simply the deviations from a straight line re-plotted on a magnified scale.

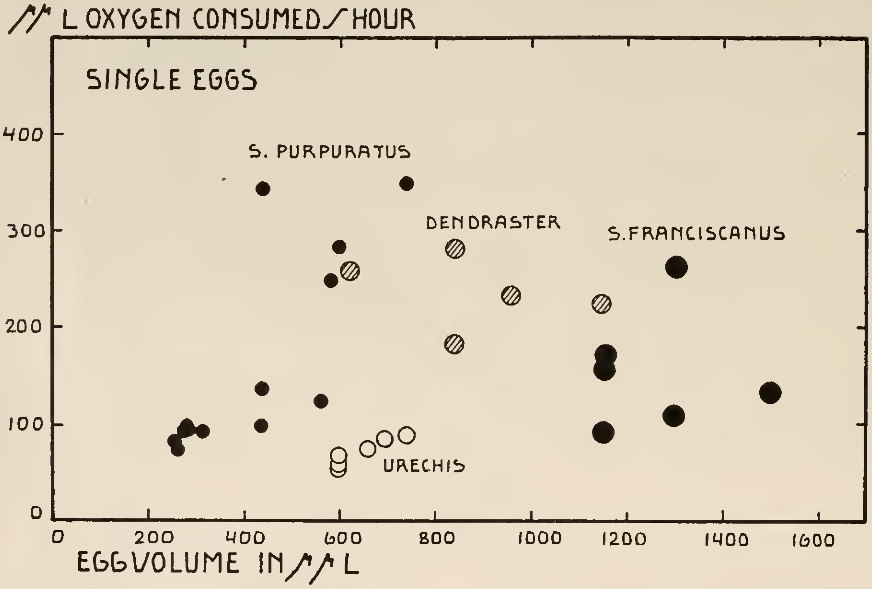

FIGURE 2. Cell volume in relation to rate of oxygen consumption in single marine eggs through the first three to four cell divisions after fertilization. Each point represents data from one egg. Tp. 15°–17°.

The results of the oxygen consumption determinations in four species of marine eggs are given in Figures 3–6. At each division the first appearance of a cleavage furrow is marked with one vertical line, and the complete separation of the cells with a second line. Up to the eight cell stage these lines are quite accurate, as all the cells could be clearly seen by turning the chamber. The beginning of the cleavage at the 8–16 cell division is accurate to about one minute, whereas the end, when the last cell finished dividing, is less certain. It is quite difficult to determine accurately the starting and finishing times for the 16–32 cell division, and for the next division (32–64 cells) they are even more difficult to see.

In Figures 3–6 the rate is represented by the slope of the curves. At the average rate they run horizontal. Above the second division a point has been located which corresponds to a 10% increase in the oxygen consumption in the period

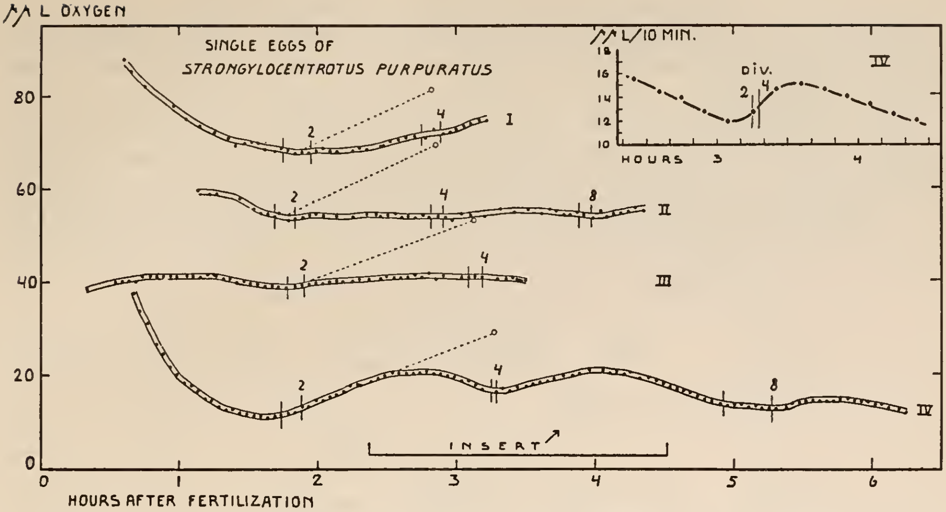

Figure 3. Deviations from a linear rate of oxygen consumption during cell division in single eggs of a sea urchin, *Strongylocentrotus purpuratus*. The average rate runs horizontal. A 10% increase of rate is indicated by the slope of the dotted lines. The ordinate units represent the absolute deviations given in μμl. The parallel lines indicate the spread of the observations and are drawn 1 μμl apart. The beginning and end of the cell cleavages are indicated by vertical lines and the digit at the end of the division gives the resulting number of cells. The position of each curve on the ordinate scale is arbitrary. Insert: Section of Curve IV recalculated as a rate curve.

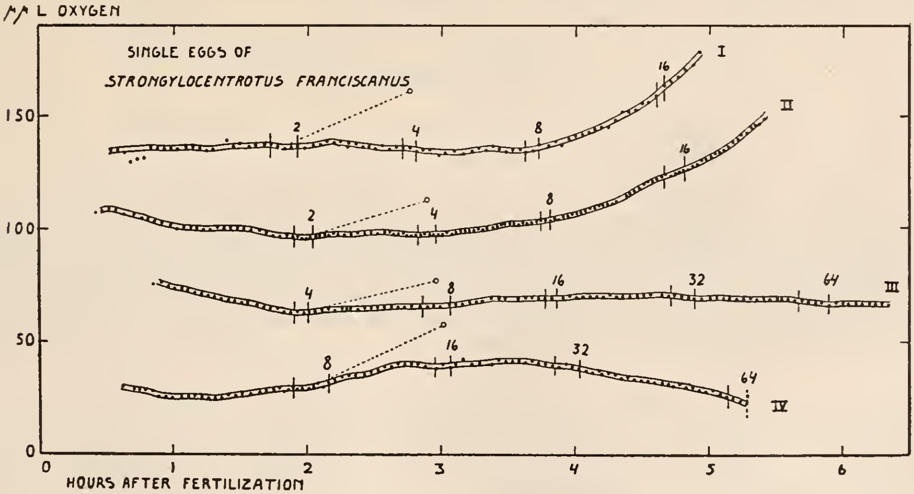

Figure 4. Deviations from a linear rate of oxygen consumption during cell division in single eggs of a sea urchin, *Strongylocentrotus franciscanus*. The average rate runs horizontal. A 10% increase of rate is indicated by the slope of the dotted lines. The ordinate units represent the absolute deviations given in μμl. The parallel lines indicate the spread of the observations and are drawn 2 μμl apart. The beginning and end of the cell cleavages are indicated by vertical lines and the digit at the end of the division gives the resulting number of cells. The position of each curve on the ordinate scale is arbitrary.

between the first two divisions, and the slope of a 10% rate change is given by the dotted line. The magnitudes of observed changes in rate can hence be estimated by comparing them with the slope of this line. The absolute deviations are directly plotted as ordinates.

Two parallel lines have been drawn one on each side of the observed points, encompassing their scattering. The distance between these lines and hence the scattering varies from 0.5 μμl to 2 μμl. Some of the curves show a slow increase of rate throughout the experiment. We dare not attach significance to these slow trends as they are in magnitude inside the possible limits of a base line drift (Scholander *et al.*, 1952a, Fig. 7), and the possibility of infection cannot be entirely discounted. A slight steady drift does not, however, obscure the relatively short-time details in which we are interested.

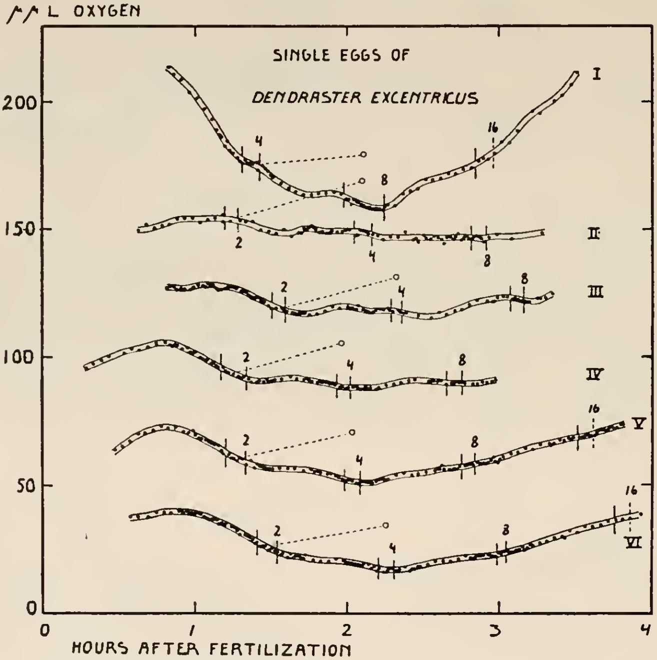

FIGURE 5. Deviations from a linear rate of oxygen consumption during cell division in single eggs of a sand dollar, *Dendraster excentricus*. The average rate runs horizontal. A 10% increase of rate is indicated by the slope of the dotted lines. The ordinate units represent the absolute deviations given in μμl. The parallel lines indicate the spread of the observations and are drawn 2 μμl apart. The beginning and end of the cell cleavages are indicated by vertical lines and the digit at the end of the division gives the resulting number of cells. The position of each curve on the ordinate scale is arbitrary.

RELATION OF EGG VOLUME TO OXYGEN CONSUMPTION

The egg cell diameter, not including the fertilization membrane, was measured, and from this the volume calculated, considering the cell a sphere. In *Urechis* the measurement was taken after the caved-in pole had swelled out. In Figure 2 the egg volume is plotted against the average oxygen consumption during the first three to four cleavages. It will be seen that there is a very considerable variability in individual eggs as to both size and rate. In *S. purpuratus* the oxygen consumption varied two- to threefold for the same egg volume and there is on the whole poor or no correlation with size. *Paramecium* with the same range of size as these eggs had a metabolic rate about 5–10 times higher (Scholander *et al.,* 1952b).

Published data from mass runs for the most part fit well with our data. Tyler and Humason (1937) found an average O_2 consumption of *Dendraster excentricus* of 208 $\mu\mu$l/hour at 15°. Lindahl and Holter (1940) gave an average egg volume of 584 $\mu\mu$l and an O_2 consumption (18°) of 155 $\mu\mu$l for *Paracentrotus lividus.* Borei (1948) obtained an average egg volume of 556 $\mu\mu$l for *Psammechinus miliaris,* with an O_2 consumption (18°) of 184 $\mu\mu$l. He found *Asterias glacialis* to have a very large egg of 2520 $\mu\mu$l with an O_2 consumption (18°) of only 250 $\mu\mu$l. *Arbacia punctulata,* with an egg volume of 190–260 $\mu\mu$l (Tang, 1931), has an oxygen consumption between 34 and 100 $\mu\mu$l (Krahl, 1950).

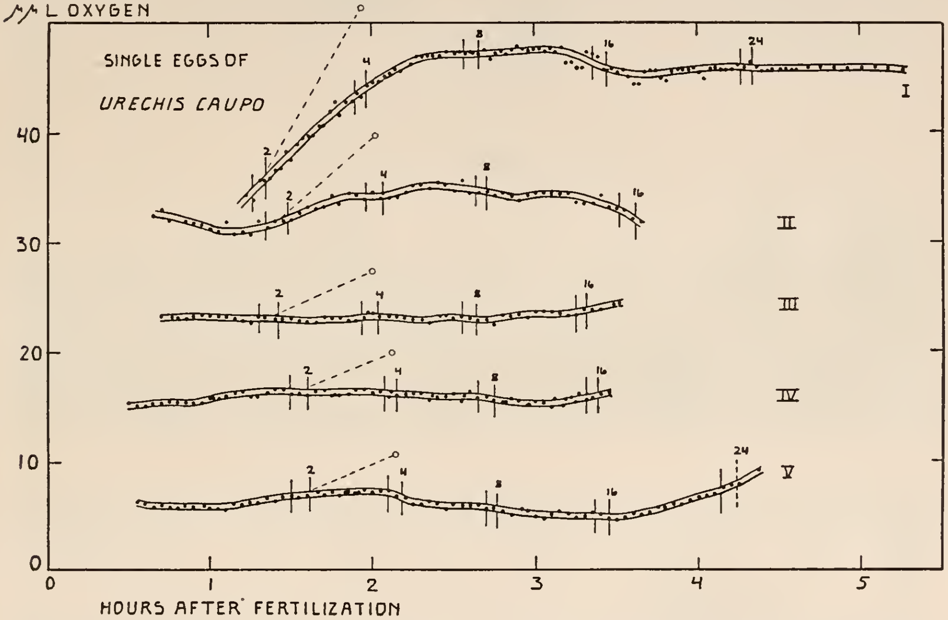

FIGURE 6. Deviations from a linear rate of oxygen consumption during cell division in single eggs of an echiuroid worm, *Urechis caupo.* The average rate runs horizontal. A 10% increase of rate is indicated by the slope of the dotted lines. The ordinate units represent the absolute deviations given in $\mu\mu$l. The parallel lines indicate the spread of the observations and are drawn 0.5 $\mu\mu$l apart. The beginning and end of the cell cleavages are indicated by vertical lines and the digit at the end of the division gives the resulting number of cells. The position of each curve on the ordinate scale is arbitrary.

The oxygen consumption in our material of single fertilized eggs varies greatly

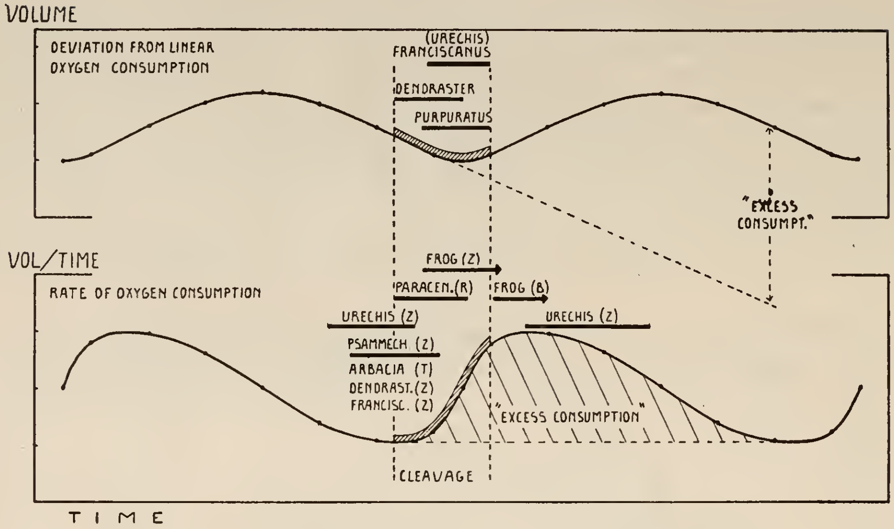

Figure 7. Generalized curves of cycling in the oxygen consumption of marine eggs. Upper: Curve showing the deviation from a linear rate of oxygen consumption such as obtained in the present investigation (Figs. 3–6). The region where cleavage occurred is given by horizontal lines. "Excess" oxygen consumption is indicated by a distance. Lower: This curve represents the upper curve recalculated as a conventional rate curve. The horizontal lines represent the regions where cytoplasmic cleavage started and ended, as found by different authors (B = Brachet, R = Runnström, T = Tang, Z = Zeuthen). The arrows (frog data) denote the region where the furrows started. "Excess" oxygen consumption is indicated by the shaded area.

individually with a poor or no correlation to size, and on the whole it seems doubtful that a near linear relation such as indicated by Smith and Kleiber (1950) exists.

Relation of Oxygen Consumption to Cell Cleavage

The deviations from an average linear oxygen consumption have been plotted for fertilized eggs of four marine species (Figs. 3–6). Data pertaining to these runs and not given in the figures are presented in Table I.

Strongylocentrotus purpuratus. This species has very slowly cleaving eggs. Figure 3, curve IV shows a considerable cycling with total rate changes of about 20–25%. In this run the third cell division seemed delayed and showed poor synchronization, leaving one cell undivided. The cyclings decrease strongly in amplitude with time. If we interpret the increase of rate after, for example, the second division, as an "oxygen debt", it would, according to the construction given in Figure 7, amount to about 10–15% of the oxygen consumed during the period between the divisions. The rate increase by the cell division may well have other causes. In the insert on Figure 3 we have given the data before, during, and after the second cell division, recalculated from curve IV of Figure 3 as a conventional rate curve. The abrupt increase of rate at the cell division and the subsequent slow decline are clearly visible. We have not been able to find any clue from the data presented in Table I as to why this egg, IV, showed such a strong cycling.

Curve III shows an increase of rate at the first cell division of about 8%, suggestive of cycling. Curve II shows also an increase of rate at the cell divisions. Curve I exhibits a steadily increasing oxygen consumption and is inconclusive as to cycling.

S. franciscanus. In Figure 4, curves II, III, and IV, there are slight rate increases at the first and second divisions suggestive of cycling, but later there are none. When the single cell in curve III divided directly into four cells there was about a 15% increase in rate, or about twice that seen in II where the egg cleaved only into two. In curve IV the single cell sprang directly into eight cells with no more increase, however, than the cell which cleaved into two. It would seem likely that splitting into eight would represent more of a disturbance than splitting into two, and that it would show up in the oxygen consumption if the eggs were otherwise the same. This discordance may be explained by the fact that different eggs may

Table I

Oxygen consumption in marine eggs

Data pertaining to Figures 3–6

No.	Temp.	$\frac{\text{mm. Hg}}{\text{min.}}$	Factor $\frac{\mu\mu\text{l } O_2}{\text{mm. Hg}}$	$\frac{\mu\mu\text{l } O_2}{\text{hour}}$	Egg vol. $\mu\mu$l	Water vol. $\mu\mu$l	$\frac{\text{Water vol.}}{\text{Egg vol.}}$	$\frac{\text{Water vol.}}{\text{Bubble vol. of } 200\ \mu\mu\text{l}}$
Strongylocentrotus purpuratus								
I	16°	0.9	1.8	98	280	40,200	144	201
II	16°	1.9	1.3	140	440	20,900	48	105
III	16°	2.7	0.6	98	280	11,800	42	59
IV	16°	1.6	0.9	83	255	18,700	74	94
S. franciscanus								
I	16°	1.4	3.4	267	1300	84,600	65	423
II	16°	2.0	1.4	165	1150	33,500	29	168
III	16°	1.9	1.0	111	1300	22,400	17	112
IV	16°	1.9	1.5	171		36,200		181
Dendraster								
I	15°	1.2	4.0	282	840	97,600	116	488
II	15°	1.2	3.4	252	600	81,500	136	408
III	15°	1.1	2.8	175		65,100		326
IV	15°	2.4	1.7	246	1150	37,800	33	189
V	15°	2.2	2.0	261		45,000		225
VI	15°	2.2	1.8	234	960	40,200	42	201
Urechis								
I	17°	1.0	1.5	90	740	33,600	45	168
II	17°	1.0	1.3	78	660	29,200	44	146
III	17°	1.1	1.1	69	600	26,200	44	131
IV	17°	0.9	1.1	61	600	26,200	44	131
V	17°	1.1	0.8	55	600	17,800	30	89

cycle to a very different degree (*cf., S. purpuratus*). The later cell divisions in both runs, III and IV, were of normal appearance. At the end of these runs the cells were taken out of the chamber and placed in dishes, where they developed into perfectly normal-appearing plutei.

Dendraster excentricus. This is the only egg tested that seems to cycle regularly. The cycling is strongly damped and almost vanishes after the third division (see Figure 5, V and VI). The cleavage takes place after a low rate and is immediately followed by a rise. In curves I, V, and VI the cycling is superimposed upon a general increase in the oxygen consumption, which gives the impression of a persisting, stepwise increase at each division. It cannot be definitely excluded that this general increase may have been caused by baseline drift or possibly infection. Most cleavages were recorded with observations every minute, in order to see if any relatively rapid changes would take place immediately at the cleavage. No such changes were found.

Urechis caupo. This animal has a slowly metabolizing egg and great care was taken to minimize the spread of the observations, which in the later runs (Figure 6, III, IV, and V) were within 0.5 $\mu\mu$l. No cycling could be observed except in number II, where it appears rather clearly.

As a result of the above described runs on single eggs we may say that the cell division in many cases takes place without any visible change in rate of oxygen consumption. When a change does occur it is most commonly seen as a rather abrupt increase of rate at the first cell cleavage. Sometimes this increase subsides and is repeated at the next cleavage. We then get cycles in the oxygen consumption. These were clearly present only in *S. purpuratus* and *Dendraster*, and of inconstant occurrence in both. The cycles have a shape usually like the one drawn in Figure 7 (upper), rather than a regular sine shape. This generalized curve has been recalculated into a rate curve (Fig. 7, lower). It will be seen that this implies that the rise of rate is much more rapid than the decline (*cf.* insert of actual curve in Fig. 3). The cycling is in all cases strongly damped and usually vanishes after the second or third division. What happens after the fourth or fifth division we have not followed.

In the cases where we see a clear cycling in the O_2 consumption it seems that the cytoplasmic cleavage is closely associated with an increase in the rate of oxygen consumption (shaded line in Fig. 7). The elevated rate may persist for 30 minutes to one hour after the division is through and has been termed an "excess consumption." It may reflect an oxidative debt left over from the cleavage, although this took place under perfectly aerobic conditions. Direct calorimetry has so far not yielded sufficiently detailed information to clarify this point. Rogers and Cole (1925) found a 10% drop in the rate of heat production at the first cleavage of eggs of *Arbacia punctulata.* Trurnit (1939) found that eggs of *Psammechinus miliaris* cleaved during a temperature maximum. His technique has been questioned (Zeuthen, 1946).

The earlier data on mass runs where cycling has been found appear on the whole to agree with our findings, in that most authors have observed cleavage to start at or near a low oxygen consumption (Fig. 7). Zeuthen (1949, 1950c) states for all his marine eggs that the cleavage furrow appears when the respiration is decreasing. His drawings actually show a relation more like that presented in our

Figure 7. By comparing his Figures 1 and 3 (1950c), the cell division in *Urechis* evidently did not correlate well with the cycling, but occurred as we have plotted it in Figure 7, one position seemingly contradicting the other. He also found periodicity in a frog egg that did not divide, and hence considered that the extra oxygen consumption was not closely connected with the cytoplasmic cleavage. We have observed two *S. purpuratus* eggs that did not divide and these did not show any change in O_2 consumption at the time when they should have normally divided.

Influence of Low Oxygen Tension Upon the Respiratory Rate and Cleavage

Experiments were made to find out to what extent lowering of the oxygen tension during an experiment would influence the rate of oxygen consumption. For this purpose the water was saturated with air instead of oxygen, so that the egg would actually run itself out of oxygen. Figure 8 shows that the oxygen consumption keeps on at normal rate until the last one or two minutes, when it abruptly stops. If the egg uses 180 $\mu\mu$l/hour or 3 $\mu\mu$l/min., and uses up all of the oxygen, then the consumption would not start to drop until there are only 6 $\mu\mu$l left. Six

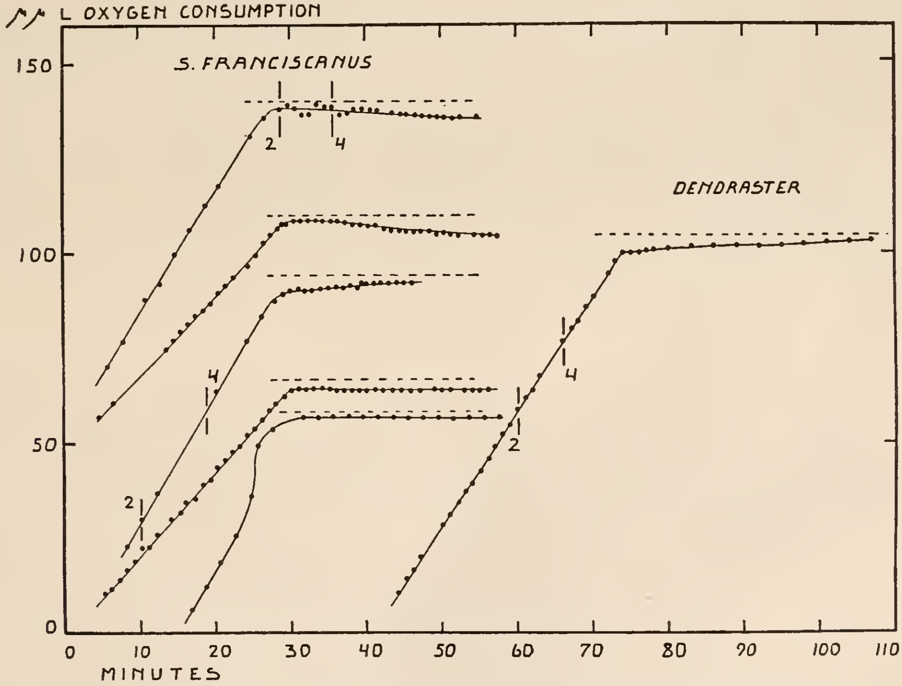

Figure 8. Oxygen consumption in single cells: experiments where the chamber water was charged with air instead of oxygen. At the sharp bend the cell has used up all of the oxygen. In the upper curve the oxygen ran out just as cell cleavage started. The cleavage continued without any oxygen consumption. The irregularities during this division are artifacts due to sticking of the pressure control, produced by the excitement of the operator. The position of each curve on the ordinate scale is arbitrary.

$\mu\mu$l oxygen will exert a pressure of 1/100 atmosphere when dissolved in 20,000 $\mu\mu$l water, and hence the oxygen consumption did not start to drop until a pressure of 7–8 mm. Hg was reached. This is in good agreement with Amberson's data on *Arbacia punctulata* (1928). In this species normal respiration keeps up until about 20 mm. O_2 tension is reached. The abrupt cessation of the oxygen consumption in our experiments is satisfactorily explained by the low O_2 capacity of the system compared to the relatively high rate of O_2 consumption.

Two of our curves showed a slight increase in flotation pressure after the cessation of oxygen consumption. This may be a sign of fixed acid formation with consequent release of CO_2. Perlzweig and Barron (1928) found lactic acid formation in *Arbacia punctulata* and Runnström (1933b) and Borei (1934) found fixed acid formation at fertilization in two other echinoderms. In two other curves we found, however, a slight decrease in the flotation pressure.

In one instance (Fig. 8, upper curve) the cleavage furrow appeared during the very minute when the egg ran out of oxygen. The division went on to full completion, however, in spite of a complete cessation of the oxygen consumption. Evidently, therefore, cell division in this species can be completed without oxygen consumption and in spite of a presumably very low oxygen tension. E. B. Harvey (1927) showed that complete absence of oxygen would stop the mitosis in *Arbacia*, and likewise in *Echinus microtuberculatus* and *Strongylocentrotus lividus*. Amberson (1928) found *Arbacia* eggs unable to divide below an oxygen tension of 4 mm. Hg. *Arbacia punctulata* will continue cleavage after the addition of KCN (Blumenthal, 1930) and Örström (see Runnström, 1933a) found that *Paracentrotus lividus* would continue cleavage if brought into a pure nitrogen atmosphere at or after the diaster stage. Our observation may therefore be taken as a direct demonstration of what previous results have strongly indicated, namely, that cytoplasmic cleavage can proceed without oxygen consumption. The anaerobic energy may well be furnished by adenosine triphosphate, as Barnett (1951) found that this substance would produce cleavage in *Arbacia* eggs that had been stopped by anoxia.

CONCLUSIONS

The presented facts may be interpreted along the following lines. The cytoplasmic cleavage is associated with a relatively sudden transformation of energy. Since the cleavage, when started, will proceed anaerobically, the energy needed is not supplied directly by oxidation. As indicated by Barnett (1951) it may be provided by the breakdown of adenosine triphosphate. The increased oxygen uptake at or following the cleavage would then be indicative of a restoration of the ATP. Therefore the actual time interval of the extra energy demands of cleavage may be much shorter than the period of extra oxygen consumption. Since the conditions are at all times thoroughly aerobic, the rate of oxidative recovery would seem to be limited rather by the amount of oxidative enzymes present in the egg than by an inability to obtain adequate supplies of oxygen. It is possible that cycling may depend upon a certain excess of enzyme, not present in a non-cycling egg. It may also be that the excess oxygen consumption reflects directly the energy requirements of the mitotic nuclear processes that take place throughout this period before the next cleavage. Some of these processes may well be associated with an increased energy exchange and could hence produce cycling of the oxygen consumption even if the cytoplasmic cleavage failed to occur, such as observed by Zeuthen (1946).

In our experiments the cycling is always strongly damped. It almost vanishes after the two or three first divisions. Zeuthen (1949) has found that later on cycling increases very markedly. Even if it can be demonstrated that this late cycling also occurs in single eggs, there will still be a minimum of cycling after the second or third division, and hence there can be no simple correlation between the wave amplitude and the steadily increasing number of cell divisions. It seems reasonable to believe that in later divisions the synchronism gets progressively poorer, and the exact nature of the late cyclings, therefore, needs further clarification. We must emphasize, however, that cell division in individual cells very often takes place without any demonstrable cycling. Quite regularly, therefore, the energy requirements for the different phases are apparently fitted nicely together within the limits of a steady constant flow of oxidative processes. It seems necessary to await much more detailed information before we can form a consistent picture of the energetics of cell division.

We wish to express our appreciation to the officers of the Milton Fund for making the above work possible, and to Dr. A. Baird Hastings we extend our sincere thanks for his unfailing interest and inspiration. We are indebted to Dr. Eric G. Ball for advice on the interpretation of the data. Dr. T. G. Thompson at the Friday Harbor Oceanographic Laboratories of the University of Washington supplied us with every possible assistance during our stay, as well as many helpful suggestions. We acknowledge with gratitude the cooperation of Dr. E. F. Swan, Dr. A. W. Martin and Dr. R. L. Fernald, from whose experience in marine biology we benefitted greatly at Friday Harbor. Much credit is due to the efficient assistance of Mr. John Stevens in procuring equipment. We are much indebted to Dr. L. R. Blinks for providing us with excellent working facilities at Hopkins Marine Station, to Dr. A. C. Giese at the same station for invaluable assistance and advice on the handling of *Urechis,* and to Dr. C. B. Van Niel for inspiring discussion.

Summary

1. In the present investigation we have analyzed the oxygen consumption during the first two to five cell divisions in single eggs from three echinoderms (*Strongylocentrotus purpuratus, S. franciscanus, Dendraster excentricus*) and from the echiuroid worm *Urechis caupo,* employing the reference diver technique (Scholander, Claff and Sveinsson, 1952a).

2. There is poor correlation between the cell volume and the oxygen consumption in individual eggs, both of which vary considerably.

3. The oxygen consumption during cell division may proceed without measurable change in rate (usual in *Urechis, S. franciscanus*) or it may show cycling correlated with the cell divisions (*Dendraster* and *S. purpuratus*). If cycling occurs, the cytoplasmic cleavage is associated with an abrupt rise in the oxygen consumption.

4. The cycling, if present, is strongly damped and often disappears after two to three divisions.

5. When started, cleavage can proceed without oxygen consumption (*S. franciscanus*).

6. Our runs on single eggs reflect in some respects the results gained by mass runs, but we are able to supply a more exact picture with respect to timing, curve shape, and variations in individual cells.

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THE MECHANICS OF FLIGHT MOVEMENTS IN DIPTERA[1]

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Although the muscles that move the wings in flies and certain other insect groups are activated by the central nervous system, the individual wing movements are not. The wing rate is determined by the load on the flight muscles and was found to bear no relation to the action potentials in these muscles (Pringle, 1949; Roeder, 1951). Two theories have recently been proposed to explain the peripheral control of wing rate in flies. According to Pringle (1949) nerve impulses produce an alteration in the flight muscles so they may be excited on being stretched. By means of the thoracic skeletal connections between them, the shortening of one set of muscles stretches, and so excites, the antagonistic muscles. Boettiger and Furshpan (1950) considered the antagonistic muscles to be under constant tetanic stimulation during flight, the mechanical action at the articulation altering the muscle load so as to produce the oscillation. Both theories suggest that the mechanical action of the thorax controls the physiological response of the muscle.

In spite of the considerable morphological work on the thorax of the fly, relatively little attention has been given to the movements of the articulation induced by the contraction of the large indirect flight muscles. The studies of Lowne (1893) and of Ritter (1911) on the blowfly are the most complete. Mihalyi (1935/36), studying the house fly, has extended the interpretations of Lowne and Ritter to include a more accurate description of some of the mechanical features of the thorax and their relation to flight.

Although Lowne (1893) gives the most detailed description of the articulating structures, in several important aspects his interpretation of their action was in error, for his methods did not show how the parts changed position during flight. The study of living flies treated with CCl_4 has revealed these movements of the articulation and enabled us to obtain a better understanding of flight mechanics. No attempt is made here to produce a complete morphological study. Most of the structures discussed are illustrated in the papers referred to above. Our purpose is to determine how the mechanical features of wing movement are related to the operation of the neuromuscular mechanism.

THE ACTION OF CCL_4

In a short abstract (Boettiger and Furshpan, 1950) the effect of placing flies in CCl_4 fumes was described. There are first violent muscular spasms involving the whole body, with an apparent stiffening of the thorax. Usually anaesthetic flight, a well recognized phenomenon in certain insects, appears. With increasing

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action of the CCl_4, the flight tone (wing frequency) increases; then it becomes lower and irregular. Suddenly the wings stop in either the up or down position, usually the up position. That this abrupt stop is due to a mechanical inhibition to movement can be shown quite readily. If the fly is immediately removed from the CCl_4 and the wings moved toward the horizontal position, they will be pushed beyond the catch and will start to beat normally again. After longer exposures, normal movements will not start when the wings are depressed. Instead, when a critical point is reached, the wings will click without further effort into the opposite position and remain locked. In this movement the wings essentially follow the normal flight path with proper changes in angle of attack, indicating that the articulation is set by CCl_4 in the same manner as in normal flight. The most effective way to move the wings down from the up position is by pressure upon the scutellum. Pressure upon the posterior lateral region of the scutum will click the wings up. Small movements of the scutellum in a fly under CCl_4 will produce much larger movements of the wings than rather extensive squeezing of the thorax of an untreated fly.

Evidence was presented (Boettiger and Furshpan, 1951) that this click phenomenon is part of normal flight, and so the action of CCl_4 becomes a new tool for studying the mechanics of flight. The wings can be frozen in the up or down position and the relations of the parts studied. Furthermore one can click the wings up and down, thus revealing the dynamic aspects of the mechanism.

All observations reported here have been made on the large flesh fly, *Sarcophaga bullata* Parker. Other species of Diptera studied gave the same response to CCl_4, but the anatomical details of their articulations have not been investigated.

The Action of the Principal Parts of the Flight Mechanism

The scutellar lever and the action of the indirect muscles

The dramatic movement of the wings produced by pressure on the scutellum of a fly whose wing articulations are set with CCl_4 depends upon the action of the scutellar lever illustrated in Figure 1. This consists of the scutellum (a) and two lateral anteriorly projecting arms (b). These are strongly attached to the scutellum by the scutellar bridges (s) and the thickened ventral triangular pieces (t) hollowed out for articulation with the postnotum. The lever rests upon two earlike lateral projections of the postnotum which fit into special grooves (r) to make an articulation allowing rotation. Between these points the scutellum connects with the postnotum by a flexible membrane. The attachment of the lever dorsally to the main part of the notum is through the scutoscutellar suture and the thin flexible cuticle (l-h, Fig. 1) along the lateral arms extending to the clefts between the lever arms and the lateral scutum. The ventral border of each lateral arm is greatly thickened and bears the posterior notal process (not illustrated). The lateral arm extends to the axillary region on each side as a process (c). The x-shaped first axillary sclerite (e, Fig. 3C) sits upon this process. The outer ventral process of this sclerite fits into a special groove (u, Fig. 1C) in which it slides as the axillary sclerite twists on process (c).

The scutellum is so constructed that the movements of the lateral arms are identical. Damage to the thin cuticle forming the dorsal surface of the scutellum allows the two wings to be manipulated up and down somewhat independently. Flies so treated cannot sustain themselves in free flight.

The arms of this lever were called the great alar apophyses by Lowne (1893) and given equal importance with the anterior notal process (p, Figs. 1 and 2) in moving the wings. Ritter (1911) also described the action of the lever arm and termed it the postalaris, the anterior notal process being the prealaris. The terms anterior and posterior tergal levers were used by Mihalyi (1935/36) who also described the rotation at the junction of notum and postnotum. These investigators all recognized the opposing action of the anterior notal process and the lever arm. In recent English literature the nomenclature of Snodgrass (1927) has been adopted. He does not describe the action of the lever arm. Pending some settlement of the

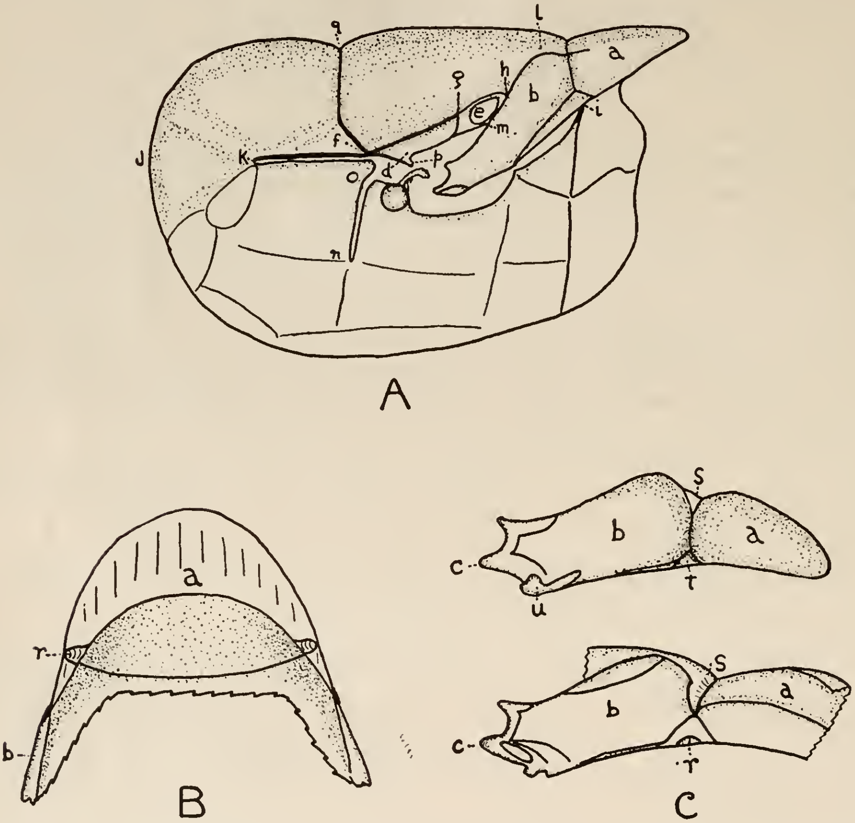

FIGURE 1. A. Left side of thorax of *Sarcophaga bullata*. B. Ventral view of scutellar lever dissected free from the thorax. C. Detail of lever arm and attachment to scutellum, outside above and inside view below. a, scutellum; b, lever arm; c, process articulating with the first axillary sclerite; d, anterior parascutum; e, posterior parascutum; f, junction of prescutal ridge, transverse ridge and parascutal hinge; g, end of parascutal hinge; h, point in line of attachment of notum with lever arm; i, point of rotation of the scutellar lever on the postnotum; j-k, fulcrum of the notum lever in the action of vertical muscles; l-m, line of attachment of notum with lever arm; n-o, lateral vertical cleft; p, anterior notal process; q, transverse ridge; r, articulating groove for attachment of postnotum; s, scutellar bridge; t, triangular structure supporting articulation of lever and postnotum; u, groove for a process of first axillary sclerite.

nomenclature by morphologists, the term scutellar lever is used here as it best describes the action of this part of the notum.

The scutellar lever by its simple rotary action and strong construction is designed to transmit the power of the indirect muscles to the wing root sclerites. In the downstroke the longitudinal muscle moves the hinge points (i, Figs. 1 and 2) forward, closing the lateral vertical clefts (n-o, Fig. 1A), while the upward movement of the notum opens the cleft (k-f). Mihalyi (1935/36) first explained the importance of these clefts in allowing proper movement of the thoracic parts. At the same time there is also a lateral expansion of the notum, especially at f (Figs. 1 and 2). The notum rides up and back as the hinge points (i) move forward; the scutellum tips down, and the lever arms move upward. If there is a definite fixed axis on which this rotation occurs, it must lie between points (i) and (l) for (i) moves forward and (l) moves back.

The part played by these structures in the upstroke is as follows. The vertical muscles reverse the rotation produced by the longitudinal muscle. In this action the whole notum anterior to the scutoscutellar suture serves as a lever with fulcrum at j-k (Fig. 1A) and moves the scutellar lever down by action at the attachment line (l-h-m, Fig. 1A). The first dorsoventral muscles shorten very little and move the lever at considerable mechanical disadvantage. They are effective, however, in producing lateral expansion at point f and stiffening the thorax. The second dorsoventral muscles act upon the lever with greater mechanical advantage and may also produce lateral expansion. The oblique muscles attach to the notum just medial to the lever arm at point L (Fig. 1A), and, although they are small muscles, must be quite effective in rotating the lever arms down. The vertical muscles, especially the oblique muscles, are arranged to pull posteriorly as well as downward and so tend to force the hinge points (i) back, opening again the vertical clefts (n-o) without at the same time straining the fulcrum region (j-k).

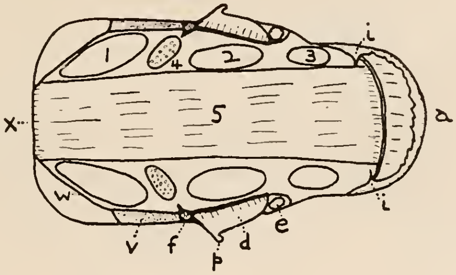

FIGURE 2. Horizontal section through the notum. Structures labeled as in Figure 1 with the following additions: v, prescutal ridge; w, chitinous supporting structure; x, anterior hardened plate; 1, first dorsoventral muscles; 2, second dorsoventral muscles; 3, oblique dorsal muscles; 4, tergal remotor muscles; 5, longitudinal muscles.

The generally accepted theory of the production of the up and down movements of the wing suggests that the longitudinal muscles expand the notum laterally on the downstroke, and the vertical muscles draw the notum inward on the upstroke. The recognition of the part played by the scutellar lever eliminates the necessity of assuming this action of the vertical muscles. A study of the insertion of these muscles on the notum makes it unlikely that they could move the lateral notum inward. In fact Lowne (1893) suggested that the longitudinal and vertical muscles are only partly antagonistic in action, as both tend to produce lateral expansion of the thorax. There must be a continuous outward force produced by the notum and transmitted to the articulation through the anterior notal process. The significance of this force in flight mechanics is discussed in the next section.

The spring mechanism of the anterior notum

The strengthening of certain regions of the thorax was well illustrated by Mihalyi (1935/36), although he does not comment on its significance in flight. Referring to Figures 1 and 2, one can see these structural re-enforcements. Point f is joined to a similar point on the opposite side of the thorax by means of the strong transverse ridge extending over the notum. From f two parallel ridges (prescutal) connected by a stiff membrane (v, Fig. 2) arise and extend anteriorly. A supporting structure (w) connects them with the anterior boundary of the thorax where it joins a hardened plate (x). The transverse and prescutal ridges strengthen the anterior notum so it will resist any force tending to move point f inward.

The attachments of the longitudinal muscle and of the anterior vertical muscles to the anterior notum are so placed that as these muscles shorten, the thorax is put under tension and point f tends to move outward. The thorax and the muscles act together to form a powerful spring. If the outward lateral force produced by this spring is greater than any opposing force, point f, the parascutum (d), and the anterior notal process (p) move outward. As shown below, during the first part of each stroke, an opposing force produced by the scutellar lever moves point f inward, thereby storing potential energy in the spring. At a critical point in the movement of the articulation, this opposing force is suddenly removed. The recoil of the spring completes the movement of the articulation and the wing. The snap action of the scutellum in wingless flies executing flight movements, as previously described by Boettiger and Furshpan (1951), demonstrates the operation of the spring mechanism. The click phenomenon seen in living flies under CCl_4 also results from its action.

The movements of the scutellar lever and of the lateral notum are transferred to the articulating portions of the notum and to the axillary sclerites of the wing base, and thus produce the characteristic pattern of wing movement. The wing cycle then results from the interaction of the anterior parascutum and the anterior notal process, the first and second axillary sclerites, and the mesopleural wing process. Their functions will now be described.

The anterior parascutum and the anterior notal process

The anterior notal process arises, as shown in Figures 1 and 2, from the thickened outer border of a shelf-like structure, formed of the anterior parascutum (d) and the posterior parascutum (e) (terminology of Lowne). Dissecting from the inside, one can see that the anterior parascutum articulates with the main part of the scutum by a concealed hinge (f-g), which allows the shelf to be moved easily up and down. Posteriorly the shelf is connected with the lever arm at h-m. The cleft between the lever and the parascutal shelf, called the tergal cleft by Mihalyi, allows somewhat independent movement of these two structures, a fact which is important in the production of the basic movement cycle.

As noted above, contraction of either the longitudinal or the vertical muscles will produce lateral expansion of the thorax at point f. This force is transmitted to the articulation by the anterior notal process. In Figure 3 (A and B) the connection between the main part of the notum and the articulation is shown. The existence of the flexible hinge connection (h), not previously described, makes the presently accepted theory of the cause of the down movement of the wings highly unlikely. Lateral expansion of the notum cannot possibly move the articulation from position shown in Figure 3A to that in Figure 3B as this theory predicts. Expansion of the notum, laterally, would move the hinge outward. However, due to rotation occurring at the hinge, the anterior notal process would not be moved out or up and the wing would remain in the up position (see Fig. 3). Through the anterior notal process the lateral force is focused on the wing articulation. The change in direction of this force necessary to wing movement is produced not by alternate flattening and arching of the notum, but by the scutellar lever. The lever

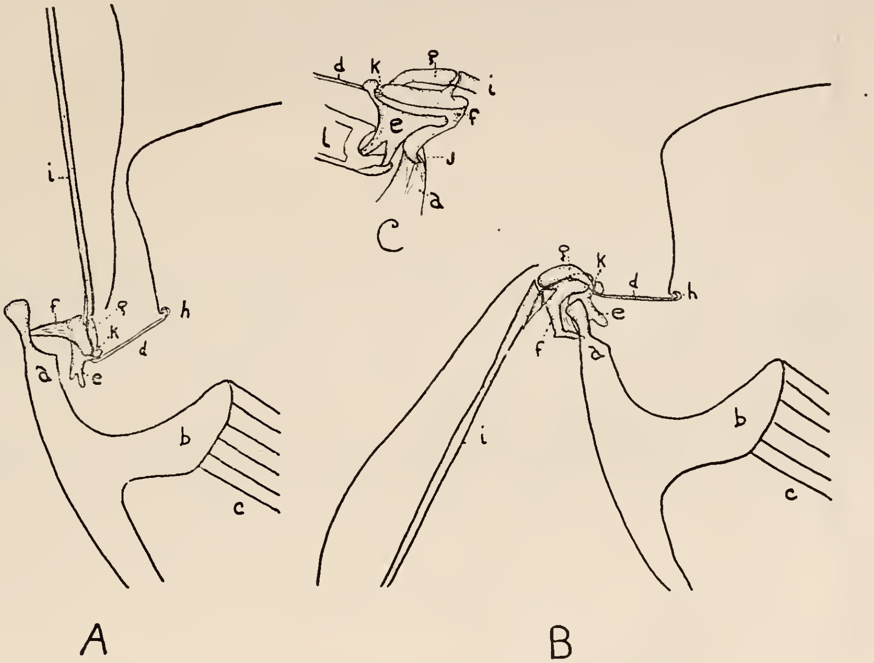

FIGURE 3. Cross sectional view of thorax showing details of the articulation of the right wing. A. Wing in up position, anterior view. B. Wing in the down position, anterior view. C. Posterior view of the axillary sclerites of right wing showing their relation to the mesopleural process, the lever arm and the anterior parascutum. a, mesopleural process; b, pleural apophysis; c, anterior pleurosternal muscle; d, anterior parascutum; e, first axillary sclerite; f, second axillary sclerite; g, base of radial vein; h, hinge; i, radial vein; j, hook articulation; k, point of articulation of anterior notal process, first axillary sclerite, base of radial vein and second axillary sclerite; l, end of the lever arm.

acts upon the anterior notal process partly through the connection between the lever arm and the parascutal shelf (m-h, Fig. 1) and partly through the first axillary sclerite (e, Fig. 3C).

The first axillary sclerite

The transformation of the movements of the lever and the anterior notal process into the complex wing cycle depends upon the action of the first and second axillary sclerites. Figure 3 (B and C) shows the relationships of the sclerites when the wings are down. The lever exerts its main effect upon the articulation through the first axillary sclerite. The large inner dorsal process of this sclerite hooks into the anterior notal process at k so that as the lever moves up and down, both k and the parascutal shelf are also moved. The second axillary lies between the two dorsal arms of the first axillary. The movement of the second axillary is largely determined by the first axillary.

The second axillary sclerite and the mesopleural wing process

Lowne (1893) described the relation between the second axillary and the mesopleural process in the blowfly. He stated that it is difficult to assign any function to this relation. Ritter (1911) believed the second axillary strengthened the joint by tying together the upper and lower parts. Snodgrass (1927) noted that the second axillary sclerite usually articulates at the side of the mesopleural process, but did not discuss the implications of this fact.

The second axillary is hooked to the side of the mesopleural process (Fig. 3C) and does not disarticulate as suggested by Lowne. The connection is a very strong one and acts almost as a universal joint to allow the complex wing cycle. The second axillary, through its relation to the radial vein, plays an important role in directing the wing. As shown in Figure 3, the base of the radial vein (g) articulates at k along with the anterior notal process and the first axillary. It also makes secondary membranous connections with the second axillary so that rotation of this sclerite moves the wings up and down.

The relation of the base of the radial vein to the second axillary is controlled in part by the direct muscles that fold the wing back over the body and bring it forward into flight position. As the wing folds back the base rotates forward. This arrangement allows some folding of the wing during flight without essentially disturbing the second axillary, the position of which plays an important role in the basic movement of the wing.

As noted above the outward force produced by the notum is transmitted to point k by the anterior notal process. This is opposed by the contraction of the anterior pleurosternal muscle (c, Fig. 3), which is one of the direct flight muscles. As this muscle draws the mesopleural process inward, a force is transmitted to articulating point k by the second axillary sclerite. The inward movement of the mesopleural process is of prime importance in setting the articulation for flight. It occurs in CCl_4-treated flies and is necessary for the production of the click phenomenon.

The position of the mesopleural process determines the range of movement of the lever arm. This is why the movements of the wings of CCl_4-treated flies are more extensive than those of untreated flies. The two lateral arms of the scutellar lever must move the same for they are strongly connected through the scutellum. Therefore it is important that the muscles exert equal forces on the two mesopleural processes. A provision for this appears to be present. The pleurosternal muscles have a common origin on the flexible sternal apophysis and they tend to act as a single muscle, the ends of which are attached to the two mesopleural processes.

The mesopleural process with its articulating second axillary sclerite opposes the lateral notum with its hinge-attached parascutum. When these structures are properly set by the action of the indirect muscles and the appropriate direct muscles, the basic movement cycle can be produced by the action of the scutellar lever. It is the action of these structures that controls the loading of the fibrillar muscles and makes possible the fast flight movements.

The Production of the Basic Flight Movements

Two conditions are required for the production of the basic flight movements: (1) the activation of the spring mechanism by tension in the indirect muscles, and (2) the inward movement of the mesopleural processes. At the beginning of flight, forces are developed to move the hook articulation of the second axillary inward, and the hinge of the parascutum, which is attached to point f, outward. In a resting fly, point k, Figure 3, is above a line connecting the hinge and the hook articulation. Consequently, as these structures approach one another, point k will move up and the wings down. Records of movements of the scutellum show that in a typical start, a small down movement, which is relatively slow, occurs first. Then the wings are suddenly and rapidly brought up. Subsequent movements occur at a frequency normal for the wing load. The flight mechanism requires a starter, and it is probable that the initial fast up movement is caused by the tergal remotor (4, Fig. 2). This muscle is a vertical muscle but is not fibrillar in structure and responds as normal tubular muscles do.

The positions of the articulating elements when the wings are in the up position are shown in Figure 3A. As the longitudinal muscle shortens, the scutellar lever moves the first axillary sclerite (e) upward. This acts on point k, which can move up only if the mesopleural process and the parascutal hinge are forced apart. By this means, potential energy is stored in the spring mechanism, to be released when point k rises above a line connecting the hook articulation and the hinge. This occurs at the critical point. The recoil of the spring drives the wings down and the articulation into the position shown in Figure 3B. On the up movement of the wings the scutellar lever brings point k down. Again energy is stored in the spring mechanism and released at a critical point.

The critical point on the downstroke occurs when point k rises above the line of force between the hook process and the hinge and on the upstroke when point k moves below this line. Referring to the discussion of the action of the indirect muscles and the movement of the notum, it can be seen that the parascutal hinge must move up on the downstroke and down on the upstroke. This movement alters the position of the line of force between the hook and the hinge. The critical point therefore is at different wing positions on the up and down strokes. The elastic recoil force takes over at least part of the muscle load before the midpoint (resting length of the muscle) is reached.

At the critical point during the movement, the recoil force is maximum, but the major component of the force is not directed to produce rotation of the second axillary. As the movement continues, however, a greater and greater component of the recoil force is in the direction of rotation, while at the same time the force is becoming progressively less as the elastic energy is used up. Vertebrates in their skeletal movements make use of this same mechanical principle (Elftman, 1941).

In flies under CCl_4 an excessive force is produced by the direct muscles (and perhaps the indirect muscles). The indirect muscles are unable to overcome the force and the wings stop in one of the two stable positions, up or down. A similar condition results from high frequency stimulation of the ganglion (Boettiger and Furshpan, 1951). The fast stops in tethered flight reported by Boettiger and Furshpan (1950) and Roeder (1951) can be explained in the same way.

THE WING CYCLE

To sustain the fly in free flight, the basic movements just described must initiate a cycle of wing movements aerodynamically designed to generate a propelling force, the magnitude and direction of which are adjustable by the insect. In this action, the indirect muscles, the direct muscles, and the structural features of the thorax are all concerned. Several authors have concluded, from the histological difference between the direct and indirect muscles, that the direct muscles cannot produce the rapid phasic changes of a single cycle. The observation that the wings of CCl_4-treated flies can be moved through a cycle by simply moving the scutellum is additional evidence for this idea, as no adjustment of direct muscle tension is possible during this movement. Mihalyi (1935/36) suggested that the indirect muscles, through the special arrangement of the three main articulating structures, can produce the complex wing cycle of straight level flight. He lists these structures as the mesopleural process, the anterior notal process and the lever arm process. The relative movements of these structures determine the movements of the axillary sclerites. These movements are able to produce a normal cycle, however, only when the articulation is set by the action of both the direct and indirect muscles.

The base of the radial vein of the wing is locked into the joint at k, Figure 3A, along with the first axillary and the anterior notal process. The important second axillary, although not articulated at k, is closely tied to this point and to the first axillary. The movements of point k govern the wing cycle. Through secondary membranous connections with the plate-like part of the second axillary, the wing moves with the rotation of this sclerite produced by the movement of k. Point k can move in the three directions, up and down with the lever, forward and back with the anterior notal process, and in and out with the rotation of the parascutum and the movement of the lateral notum.

Referring to Figure 3, one can analyze to some extent the movements that produce the wing cycle. When the wing is in the down position, it is held at the extreme forward point of the cycle in a vertical plane along side of the thorax. The articulation in this condition is shown in Figure 3 (B and C). As the vertical indirect muscles shorten, the scutellar lever process (1, Fig. 3C) starts to move down. The parascutum (d) does not move much at first, as it is held by the tension between the mesopleural process (a) and the parascutal hinge (h). Therefore, the second axillary first tips back, rotating the base of the wing so as to raise the anterior margin and depress the membranous part. As the lever continues to move down, point k moves more rapidly. The wing flips up and back in an effective stroke with the anterior margin of the wing leading. Before coming into the up position, a rotation of the wing base depresses somewhat the anterior wing margin. This is shown by the fact that in CCl_4-treated flies the wing locks into the up position with the anterior margin of the wing leading in a position to begin the downstroke. This rotation during the upstroke is due to the forward movement of point k, produced by the anterior notal process, and the twisting of the second axillary, resulting from the inward and downward movement of the lever arm.

The downward movement of the wing is also initiated by the scutellar lever as it is moved up by the shortening of the longitudinal muscles. The first axillary is lifted in such a way that the second axillary twists to tip the anterior margin of the wing down. Then point k flips up and the wing, held at the proper angle of attack, moves down. During the down stroke, the backward movement of point k and the forward movement of the lever arm process twist the second axillary, thereby insuring the full forward sweep of the wings. In CCl_4-treated flies the wing locks in the down position with the anterior margin closer to the body than the membranous portion as shown in Figure 3B. The wing is set as at the end of a downstroke, instead of ready for the upstroke. At the beginning of the upstroke the anterior margin of the wing must be raised while the membranous portion is depressed, as noted above. The upstroke is more complicated. The articulation is set to raise the anterior margin of the wing at the beginning of the upstroke and depress it at the end. Records of movements of the scutellum during a quick stop reveal a basic asymmetry in the articulation. In a quick stop on the downstroke there is some movement of the scutellum before the stop point is reached. On the upstroke the stop occurs almost at the extreme down position of the scutellum (Boettiger and Furshpan, unpublished).

The base of the wing makes secondary connections to the driving mechanism. The most important one is with the posterior notal process, which arises from the ventral border of the scutellar lever arm. This undoubtedly aids in tipping the wing and supporting the base against the effects of air pressure. However, cutting the posterior notal processes on both sides does not destroy the fly's ability to maintain itself in free flight.

The Mechanical Limits to Movement

Normally, more energy is released than is necessary to complete the movement (Boettiger and Furshpan, 1951). The freely moving lever is stopped by definite mechanical limits, which appear, however, to have some elasticity. The kinetic energy of the moving wings may be partially recovered by this means, as suggested by Chadwick (1951). The lever arm can move down without hindrance until its ventral ridge contacts the pleural sclerites. This first occurs quite near to point i (Fig. 1), as the arm is set at an angle so that it moves inward as it moves down. The second axillary sclerite seems to limit the up movement of the lever. As the lever pushes against the first axillary, the second axillary moves into the position shown in Figure 3C. The hook articulation with the mesopleural process prevents any further upward movement of the second axillary and hence of the first axillary and the lever. The presence of mechanical stops may increase the efficiency of the mechanism by eliminating the necessity for doing negative work in stopping each movement (Fenn, 1930).

The Steering Mechanism and the Control of Amplitude

The movements of the lever arms and therefore of the articulation must have the same amplitude on the two sides. The construction of the scutellum does not allow one arm to move more than the other. If the wings are not drawn forward into flight position when a fly is under CCl_4, the amplitude of the wing movement when the scutellum is depressed is quite small. Even though the wings merely tip down, the click action is present and the lever arms move the full limit. The first axillary does not twist outward and forward as it does on the downstroke when the wings go through their full amplitude. As the wings are brought forward by direct muscles, the amplitude increases. Frequently, mounted flies will show erratic movements and hold one wing back while the other moves with normal amplitude. They spin around toward the side of the smaller movement. In a similar manner, a free flying insect can alter its direction without changing the action of the indirect muscles or of the scutellar lever. Should the fly wish to rise or fall, the tension in the proper direct muscles is adjusted, altering the wing cycle equally on both sides and again requiring no change in the driving mechanism.

Chadwick and Williams (1949) and Chadwick (1951) have made a quantitative study of wing movements in Drosophila at various air densities. Reducing the density of the air lowers the wing load and increases both frequency and amplitude. The increase in frequency is proportional to the increase in amplitude. This proportionality may be interpreted to mean that the increased amplitude observed at low air density is the result of the greater momentum of the faster moving wings. With greater momentum the articulation moves further into the elastic mechanical stop. In addition, each wing will also bend more when its articulation is suddenly stopped and this would be recorded as an increase in amplitude. Changes in frequency and amplitude may, therefore, be produced experimentally without alteration in the action of the basic neuromuscular mechanism. Roeder (1951) has found that reducing muscle load by removal of the wings results in a decrease in frequency of thoracic potentials. This suggests that receptors, responsive to wing load, can exert some control over the indirect flight muscles.

The amplitude of wing movement must be controlled to some degree by the central nervous system. In the first place, the wings may be held forward or back as described above so that their amplitude is large or small. Secondly, the tension between the mesopleural process and the parascutal hinge which determines the spring action of the notum can control wing amplitude. If this tension is large, the indirect muscles must, in overcoming this force, store a great amount of energy in the spring mechanism. The recoil action will then throw the articulation to the limit and so give maximum wing movement. On the other hand, if the tension is small, less energy is stored and the recoil may not move the articulation to its limit. In a normal stop there is a progressive decrease in amplitude of wing and articulation. This is apparently due to the relaxation of the direct muscles controlling the position of the mesopleural processes. Records show that during continuous flight under constant conditions, the amplitude of movements of the scutellar lever is remarkably uniform. Occasional changs in amplitude may be accounted for by altered tension between the mesopleural process and the parascutal hinge. The indirect muscles by controlling the spring action of the notum may contribute to these amplitude changes.

SUMMARY

1. The mechanics of insect flight are usually studied by squeezing the thorax so as to imitate the action of the indirect flight muscles. In flies certain elements of the articulation are not properly set in such experiments, so the interpretations made are not accurate. That CCl_4 sets the articulations of flies as in normal flight is shown by the dramatic wing movements easily produced in these insects. This report is based on the study of CCl_4-treated flies and outlines the main features of the mechanics of wing movement. Much additional physiological evidence for the interpretations given here has been accumulated and will be reported separately. The abstracts referred to state some of the experimental results.

2. The secret of the peripheral control of wing rate certainly lies in the indirect flight muscles. These muscles are the power plants of the wings, operating in essentially an all-or-none fashion. It is shown here that they play little part in steering or in amplitude changes. They operate between definite mechanical limits and so do not halt each other's movement as in a conventional antagonistic system.

3. The anterior notum is shown to have a spring action which unloads the muscle at a critical point and so plays a basic physiological role. If the recoil of the notum alone is capable of completing the movement, the indirect flight muscles would not be required to exert any tension at their resting or shorter lengths. The energy would be put into the mechanism almost isometrically and it would not be necessary to assume high tension in a rapidly shortening muscle.

4. By a combination of the mechanical properties of the thorax and the physiological properties of the fibrillar muscle the peripheral control of wing rate is achieved.

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HORMONAL REGULATION OF THE DISTAL RETINAL PIGMENT OF PALAEMONETES

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There have been several investigations (Parker, 1897; Welsh, 1930; Bennitt, 1932; Kleinholz, 1936) of the control of the migration of the distal retinal pigment of the prawn, *Palaemonetes.* Bennitt demonstrated that when one eye of *Palaemonetes* was covered and the other eye illuminated, both eyes assumed to a greater or lesser extent the light-adapted state. This suggested a hormonal control of the retinal pigments. Later, Kleinholz (1936) induced the distal retinal pigment of *Palaemonetes* to assume the light-adapted position by injecting eye-stalk extract into dark-adapted prawns, thus suggesting the existence of a light-adapting hormone whose source was the eye-stalk. That this was the normal controlling mechanism was indicated by the fact that eye-stalks of light-adapted animals were more bountifully supplied with this factor than those of dark-adapted ones.

The results of the experiments of Brown, Fingerman and Hines (1952) and Brown, Webb and Sandeen (1952) in which dark-adapted prawns are exposed to 250 ft.-c. light flashes both with and without earlier conditioning stimuli have not only given strong support to the hypothesis that there is a light-adapting hormone normally active in regulating the position of the distal retinal pigment of *Palaemonetes,* but have also indicated the action of a dark-adapting hormone. Prawns kept in darkness overnight exhibit quite a different rate of re-dark-adaptation when returned to darkness following a one-minute, 250 ft.-c. light exposure early in the morning than do prawns which earlier the same day had received the normal stimulus of dawn illumination before being placed in darkness and later submitted to the one-minute light exposure. Such a difference was postulated to be due to different degrees of availability of a dark-adapting factor.

The present experiments were undertaken: (1) to establish more definitely the presence of a dark-adapting hormone, (2) to determine the sources of the light- and dark-adapting hormones within *Palaemonetes,* and (3) to elucidate further the normal roles of these hormones in light- and dark-adaptation.

Materials and Methods

For the following experiments specimens of the common prawn, *Palaemonetes vulgaris,* were usually collected daily from the Eel Pond in Woods Hole, Massachusetts. In the laboratory the stock supply of animals was kept in aquaria in running sea-water from which the animals for each experiment were randomly taken without regard to size or sex. The experiments were conducted during the months of June and July.

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The influence of the various experimental procedures on the distal retinal pigment in the eyes of these prawns was observed through the use of a method of direct measurement of the position of this pigment in the intact, living animal (Sandeen and Brown, 1952). The method consisted essentially of holding a prawn on the stage of a dissecting microscope and viewing the eye under a relatively high magnification using transmitted light. With an ocular micrometer the width of the transparent area, which is a direct function of the degree of light-adaptation, can be measured from the cornea to the distal margin of the pigment. Since the animals varied considerably in size, the degree of light-adaptation was always expressed as a ratio of this width to the total distance from corneal surface to the proximal edge of the retina using arbitrarily as a marker the black spot, or ocellus, which is apparent on the dorsal aspect of the eye-stalk. This ratio will be referred to as the distal pigment index.

In all experiments in which *Palaemonetes* were placed in the dark room or exposed to various light intensities, white enamelled pans with a bottom diameter of approximately 7 inches were used. Sea water was placed in the containers to a depth of about 1½ inches. To prevent overcrowding no more than 12 to 14 animals were ever placed in a single pan. The various illuminations which were used were obtained with incandescent lamps placed at adjusted distances directly above the animals. The resultant illuminations were measured with a Weston photometer.

In the experiments in which prawns were injected with extracts of various organs the extracts were prepared in the following manner. The organs were removed with the aid of a dissecting microscope, from normal animals taken from the stock supply, and transferred to a small container of sea water. When a sufficient number of the organs had been obtained they were placed with a minimum of water in a glass mortar with a finely ground surface and while still moist were triturated as completely as possible with a glass pestle. Sea water was then added to make up the desired concentration, mixed thoroughly, and the extract drawn into a one ml. hypodermic syringe graduated in hundredths. The whole procedure was carried out very rapidly and the extracts used immediately. In no case did more than 30 minutes elapse between the beginning of the dissections and the injections.

Comparison of the Responses of One-Eyed and Normal Animals

In the first series of experiments the influence of the removal of one eye on the distal retinal pigment of the remaining eye was determined. In each of six experiments five *Palaemonetes* were taken at random from the stock supply and placed in a small amount of sea water in a white enamelled pan at an illumination of 25 to 50 foot-candles. One eye-stalk was carefully removed with a pointed scalpel under a dissecting microscope and the eye-stub cauterized immediately. The position of the distal pigment of each eye to be observed in the experiment was determined prior to the operation and then at 5, 10, 15, 30, 60, 90, 120 and 240 minutes following the operation. The first three of these experiments were conducted in the morning and the last three in the early afternoon. No significant difference in responses was found for these two times of day.

The average distal pigment index of the five animals in each experiment was calculated for each time that a determination was made. These averages were used to calculate a final distal retinal pigment index for each time that a reading was made for all six experiments. Therefore, each final index at any given time represents an average of the condition for 30 animals. The final average indices of the six experiments were used to prepare Figure 1(A). It can be seen that the distal pigment index drops from 0.18 to 0.16 in 30 minutes and remains at this lower level for the duration of the experiment, a period of four hours. It had previously been determined (Sandeen and Brown, 1952) that at this illumination the distal pigment index of normal animals has a value ranging between 0.18 and 0.20. This is represented in Figure 1(A) by the broken line above the curve just described. Thus, under conditions of equal illumination the distal retinal pigment of *Palaemonetes* with only one eye-stalk is maintained at a lesser degree of light-adaptation than that in normal animals with their two eye-stalks.

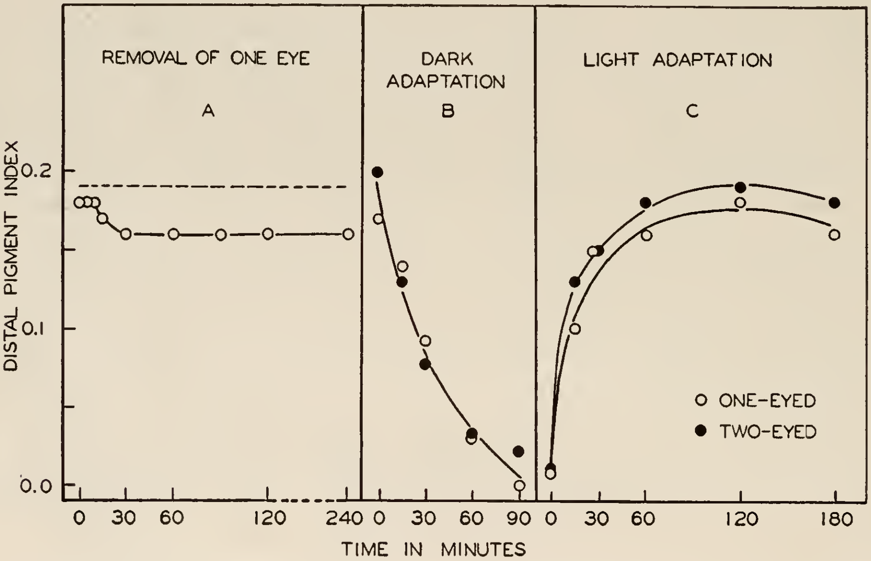

Figure 1. A. Change in the degree of light adaptation of the distal retinal pigment following removal of contralateral eyestalk. B. Comparison of rates of dark-adaptation in one-stalked and normal prawns. C. Comparison of rates and degrees of light-adaptation in one-stalked and normal prawns.

In a second series of experiments the rate of dark-adaptation of one-eyed prawns was compared with that of normal prawns. Approximately twenty-four hours before each experiment specimens of *Palaemonetes* were taken from the stock supply of animals and one eye of each was removed and the eye-stub cauterized. These animals were then returned to an aquarium with running sea water until the experiment was conducted the following day.

In each of the two experiments which were performed, 10 one-eyed *Palaemonetes* were placed in each of five pans. Similarly, normal animals were taken at random from the stock supply and 10 were placed in each of five pans. The distal pigment indices of 10 one-eyed animals and 10 normal animals in one of the pans of each group were determined. These two pans together with the others in each group were then placed in the dark room. In the first experiment distal pigment indices of one-eyed and normal *Palaemonetes* were determined at 15, 30, 60 and 90 minutes after being put into the dark. In the second experiment distal pigment indices were determined only at 30, 60, and 90 minutes. At each of these times one container from each group was removed from the dark room and the distal pigment indices of the animals were determined. Following the determination these animals were discarded; a different container of animals was used for each successive determination.

The average distal pigment index for 10 animals of each group at each time that a determination was made was calculated. Average values were then obtained for both experiments and these were used to prepare Figure 1(B). Since a distal pigment index determination was not made at 15 minutes in the second experiment, the average index shown in the graph is that obtained in the first experiment.

It can be seen from Figure 1(B) that there is no significant difference between the rates of dark-adaptation of these two groups of animals. The presence of one eye-stalk is sufficient to permit an animal to dark-adapt at the normal rapid rate.

In a third series of experiments the rate of light adaptation of one-eyed animals was compared with that of normal animals. In each experiment a group of six pans containing 10 normal animals each and a group of six similar pans with 10 one-eyed animals in each were left in darkness overnight and then brought abruptly into an illumination of 250 ft.-c. where the course of light-adaptation of the animals was followed.

The distal pigment indices of 10 normal animals and 10 one-eyed animals were determined at the time the two groups were brought into the light and at 15, 30, 60, 120 and 180 minutes thereafter. The average value at each time of index determination for three such experiments was calculated. Since the determinations in the first of the three experiments were not continued beyond 120 minutes, the average value for 180 minutes was obtained from the last two experiments.

The average distal pigment indices obtained from the three experiments for each time that a determination was made were used to prepare Figure 1(C). It can be seen from this graph that the rate of light-adaptation and the final degree achieved with one-eyed animals are less than that with normal animals. The distal pigment index reached by the one-eyed animals is quite comparable to that seen following the removal of one eye from normal animals in light. It appears, therefore, that both eye-stalks are essential for the normal rate and degree of light-adaptation of each eye under these conditions of illumination.

INFLUENCE OF A BRIEF LIGHT EXPOSURE

The extensive light-adaptation in the dark which results from the interruption of a long dark period by a one-minute exposure to light at 250 ft.-c., described elsewhere (Brown, Fingerman and Hines, 1952), suggested the operation of a light-adapting hormone. It appears that an adequate stimulus for secretion of such a substance is a brief, bright, light flash and that the activity of the substance long outlasts the duration of the original brief stimulus. Prawns with a single eyestalk and in darkness responded to a one-minute, 250 ft.-c. flash by substantially less light-adaptation than that seen in normal prawns subjected to a similar stimulus.

It was desired further to determine whether a second substance might be operating in dark-adaptation as postulated by Brown, Fingerman and Hines (1952).

Since a change from darkness to light is evidently an adequate stimulus for the release of the light-adapting hormone it seemed reasonable that a change from light to darkness would be a normal stimulus causing secretion of a dark-adapting hormone. In order to test this possibility an experiment was designed in which responses to a brief light flash were compared following different durations of dark periods.

Palaemonetes were taken at random from the stock supply and distributed among six groups of five white enamelled pans. Approximately 12 animals were put into each pan. The first group of animals constituted the control group. Distal pigment indices of 10 animals from one of the pans of this group were determined at 9 A.M. and the remaining containers were placed immediately in darkness. The course of dark-adaptation was followed by removing a pan from darkness and determining distal pigment indices of 10 animals after 15, 30, 60 and 120 minutes.

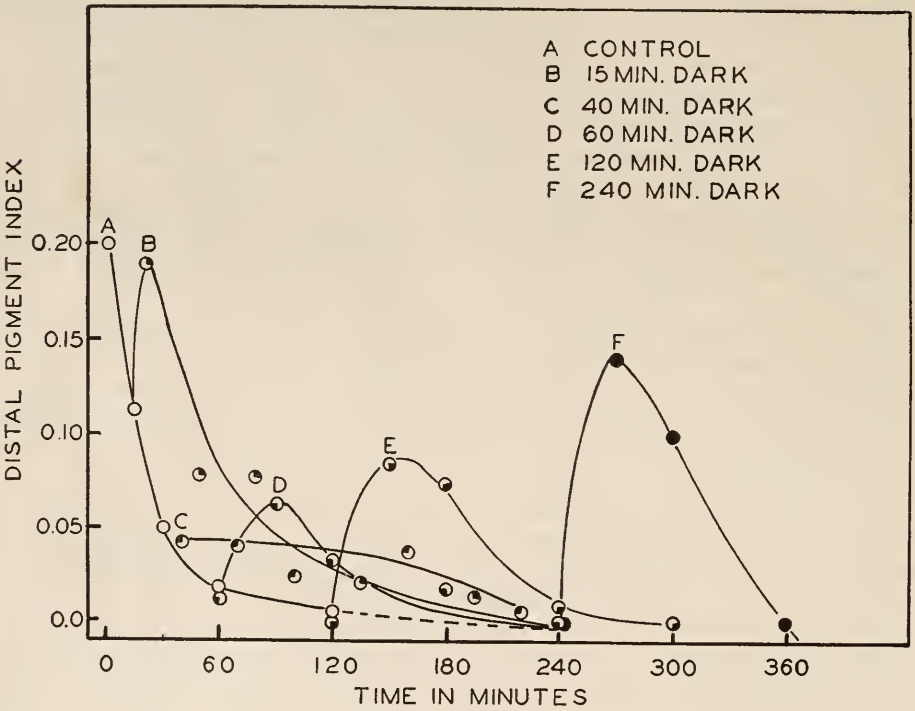

FIGURE 2. Responses of the distal pigment to a one-minute, 250 ft.-c. light flash after different intervals in darkness.

The remaining five groups of pans were also placed in darkness at approximately 9 A.M. At 15 minutes one group of five pans was subjected to a one-minute, 250 ft.-c., flash of light. Immediately following the flash the distal pigment indices of 10 animals from one pan were determined. The course of dark-adaptation of this group was followed by determining distal pigment indices of 10 animals after 30, 60, 120, and 180 minutes. Similarly, of the other groups, one was dark-adapted for 40 minutes, one for 60 minutes, one for 120 minutes and the last for 240 minutes. At the end of each of these periods the group of animals was treated in a similar manner to that described for the 15-minute group.

In all the groups for each time that a determination was made an average distal pigment index of the 10 animals was determined. These average indices were used to prepare Figure 2 where the distal pigment index is plotted against time in minutes. In Figure 2 zero time represents that time at which all groups were put into darkness for the first time.

It is apparent from this figure that both the amount of light-adaptation induced by the one-minute flash and the rate of subsequent re-dark-adaptation are influenced by the duration of the dark period. By examination of curves C, D, E, and F, representing 40, 60, 120 and 240 minutes of exposure to darkness prior to the light flash, it can be seen that the longer the period in darkness the greater the extent of light-adaptation resulting from the flash and the more rapid the subsequent rate of dark-adaptation. However, it can also be seen that 15 minutes of exposure to darkness is not sufficiently long to yield a result which is entirely consistent with this generalization. This group light-adapted during the first 15 minutes following the flash to a state almost equivalent to the fully light-adapted state shown by all groups when they were removed from the aquaria at the beginning of the experiment, as illustrated by the initial index of the control group. The rate of dark-adaptation was, however, somewhat less than that shown by the controls.

These results strongly support the hypothesis of the operation of two hormones, one inducing light-, and the other, dark-adaptation. Since greater degrees of light-adaptation result from a one-minute flash of light as the animals are left longer in darkness, it would appear that the ability to secrete the light-adapting principle increases, at least for a few hours, in darkness. On the other hand, the increase in the rate of dark-adaptation with increasing time in darkness can be explained in terms either of an increase in ability to secrete the dark-adapting principle in response to return to darkness or to the presence of a higher titer of this material in the blood for the normal maintenance of the dark-adapted state.

Injection Experiments

In an attempt to obtain some information regarding the sources of the substances which function in the light- and dark-adaptation of *Palaemonetes* distal pigment, experiments were designed in which extracts of eyestalks and of central nervous organs were injected into animals under various conditions.

The first type of experiment consisted of injecting extracts of eyestalks of *Palaemonetes* into animals which had been previously dark-adapted for a minimum of three hours. These animals were taken at random from the stock supply and five were placed in each of four white enamelled pans partially filled with sea water. The extract was prepared by triturating freshly removed eyestalks in a sufficient quantity of sea water to yield such a final concentration that each animal, receiving an injection of 0.02 ml., received the equivalent of one eyestalk. The extracts were centrifuged. The animals were injected in the dark-room under a red photographic light which had previously been tested and shown to produce no light-adaptation. At 30, 60, 120 and 240 minutes one pan of animals was removed from the darkroom and the distal pigment indices of the five animals determined. An average distal pigment index was calculated for each time that a reading was made. Two experiments of this sort were performed and the results averaged. The averages of the two experiments were used to prepare Figure 3.

It can be seen from Figure 3 that the injection of eyestalk extract produces light-adaptation comparable to that produced by the interruption of a dark period by a one-minute, 250 ft.-c. flash of light. The maximum degree of light-adaptation achieved occurs in 30–60 minutes following injection. The dark-adaptation which followed was not quite complete at the end of 180 minutes.

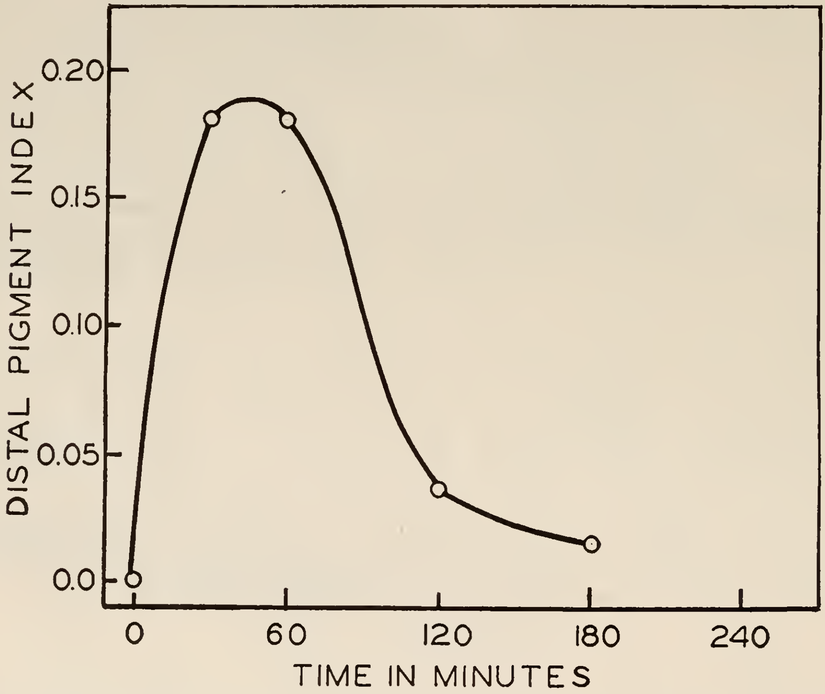

Figure 3. Response of dark-adapted prawns in darkness to injection of an extract of eyestalks.

Preliminary experiments in which sea-water extracts of various central nervous organs were injected into similarly dark-adapted *Palaemonetes* yielded inconsistent results. In some cases no light-adaptation was obtained while in others distal pigment indices of the order of 0.03 were obtained, suggesting the presence of light-adapting substance in nervous tissue.

Another method of assay of the extracts was used. A study was made of the influence of injection of extracts of eyestalks and of central nervous organs on the response of dark-adapted prawns to a brief exposure to bright light. For each experiment *Palaemonetes* were taken from the stock supply and distributed among three groups of five pans with five animals in each pan. These animals were all placed in the darkroom overnight and then about 6 the next morning brought abruptly into an illumination of 250 ft.-c. for a period of 20 minutes. During this period of light exposure one group received injection of an extract of the brain, connectives and ventral cord. The second group received extract of eyestalk, and the third received only sea water. Each animal was given a dose of 0.02 ml.

The extracts of the central nervous system were prepared by dissecting the brain, circumesophageal connectives, and thoracic and abdominal cords from normal light-adapted animals and extracting them in sea water in such an amount that an animal receiving a dose of 0.02 ml. received the equivalent of half a total nervous system. The extract was centrifuged. The extract of the eyestalks was prepared as previously described, such that each animal received one eyestalk or half the equivalent of the complement of a normal animal.

Two experiments of this sort were performed. In the first, distal pigment indices of five animals in one of the pans were determined immediately following the exposure to light while the rest of the containers were placed in the darkroom. Successive determinations of distal pigment indices were made at 30, 60, 120 and 180 minutes, using a different pan of animals for each determination. In the second experiment determinations of the distal pigment indices were made at 30, 60, 120, 180 and 240 minutes from the time the pans were returned to darkness. Averages of the distal pigment indices of five animals for each time that a determination was made were calculated and average values for the two experiments were obtained. Since the initial and 240-minute determinations were made in only one of the two experiments, the average obtained in one experiment was used as the definitive one.

The average distal pigment indices for the two experiments were used to prepare Figure 4. It can be seen from this figure that, compared with the sea-water controls, extracts of the central nervous system as well as those of the eyestalks supplement the amount of light-adaptation which occurs as a result of the exposure to light. Furthermore, there is a significant increase in the subsequent rate of dark-adaptation over that seen for the controls. The extracts, therefore, appeared to contain both light- and dark-adapting principles.

In an effort to determine whether the two hormones are differentially distributed within the central nervous system further experiments were designed in which extracts of various parts of the nervous system were compared with extracts of the eyestalks. In preliminary experiments of this sort it was found that extracts of the abdominal cord, thoracic cord, circumesophageal connectives and the brain all behaved qualitatively like an extract of the total nervous system. However, this seemed not to be true for extracts of the tritocerebral commissure.

In two experiments sea water extracts of eyestalks, tritocerebral commissures, and sea water were injected into three groups of overnight-dark-adapted *Palaemonetes* while they received a ten-minute exposure to an illumination of 250 ft.-c. at about 6 A.M. The extract of eyestalks was of such concentration that an animal receiving a dose of 0.02 ml. received the equivalent of half an eyestalk or a quarter the equivalent of the complement of a normal animal. The extract of tritocerebral commissure was prepared by removing the brain and circumesophageal connectives from several animals in such a manner that the tritocerebral commissures were not damaged. As they were removed, these organs were placed in sea water in a Syracuse watch glass until the desired number had been obtained. The commissures were then carefully removed by severing them with a scalpel at their junctions with the circumesophageal connectives. They were then transferred to a microscope slide with a minimum of water and triturated as completely as possible with a small glass pestle with sea water to yield a concentration such that an animal receiving the usual dose of 0.02 ml. would receive the equivalent of ¼ of a commissure.

Each extract, including sea water as a control, was injected into five groups of 10 animals. After the light exposure and simultaneous injection of the three groups the animals were returned to the darkroom and the course of dark-adaptation was followed by making distal pigment index determinations for 10 animals of each group at 30, 60, 90, and 120 minutes from the beginning of the light flash.

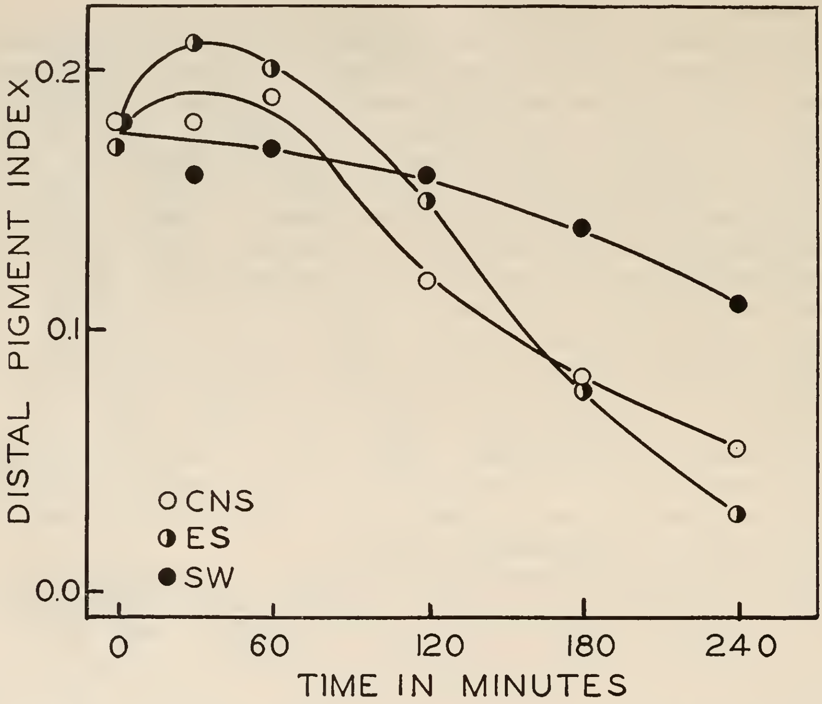

Figure 4. Comparison of the light- and re-dark-adaptational responses of prawns overnight in darkness to a simultaneous 250 ft.-c. light stimulus and injection of extracts or sea water. CNS—central nervous system; ES—eyestalk; SW—sea water.

It can be seen from Figure 5 that the injection of extract of tritocerebral commissure during the light flash resulted in a light-adaptational response which was significantly less than that achieved by the animals receiving only sea water. The animals receiving extract of eyestalks responded in a manner essentially similar to those in the previous experiments. The extent of light-adaptation was substantially greater than that shown by the sea water controls. These results suggested that in the tritocerebral commissure the dark-adapting hormone was present without the light-adapting one.

In order to establish further the character of the influence of the extract of the tritocerebral commissures a final experiment was designed. This one continued for a period of time long enough to follow re-dark-adaptation. In this experiment three groups of three pans containing 10 animals in each were given a one-minute, 250 ft.-c. flash of light at 6 A.M. and then returned to the darkroom. At the end of an hour in the dark when the animals were expected to be maximally light-adapted as a result of the flash, they were brought into 250 ft.-c. illumination again for ten minutes during which time one group was injected with extract of tritocerebral commissure, the second group with an extract of eyestalk, and the third group with sea water as a control. The extracts were prepared in the usual manner. Following injection the three groups of pans were returned to the darkroom and distal pigment indices of 10 animals in each group were determined at 30, 60, 150, 210 and 270 minutes following the beginning of the second light period. Since there were only three containers of animals in each group, each container was returned to the darkroom after each distal pigment index determination so that it could be used for another determination. Thus, container 1 was used for the 30-minute and the 210-minute determination while container 2 was used for the 60-minute and the 270-minute determination. It had previously been determined that the brief exposure to the microscope light during a determination for animals in this experimental state was not significantly effective in inducing light-adaptation. This is substantiated also by the response of the control group of this experiment.

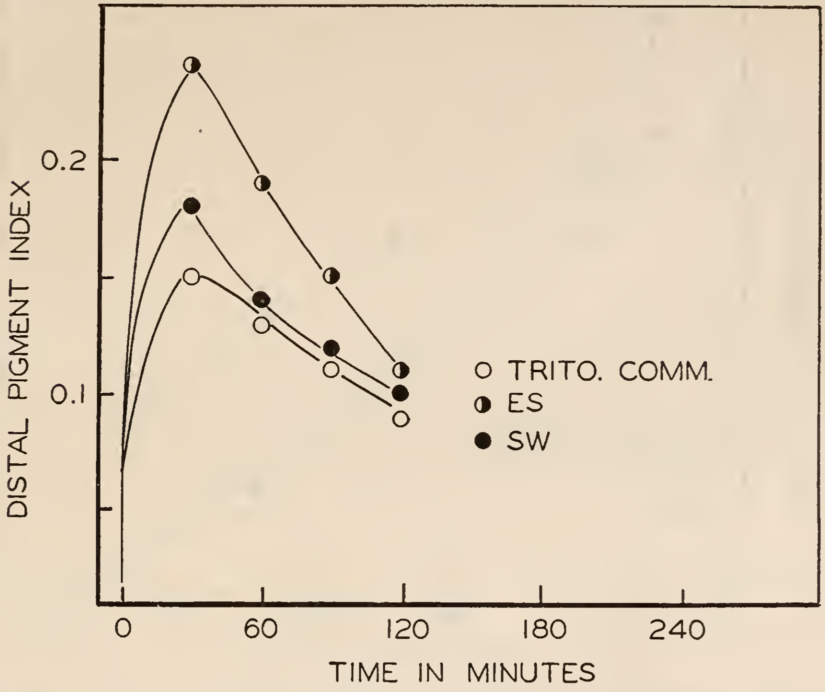

FIGURE 5. Comparison of the light- and re-dark-adaptational responses of prawns overnight in darkness to a simultaneous 250 ft.-c. light stimulus and injection of extracts or sea water. Trito. Comm.—tritocerebral commissure; ES—eyestalk; SW—sea water.

Averages of the distal pigment indices of the 10 animals for each time that a determination was made were calculated and plotted in Figure 6. Zero time in this figure indicates the beginning of the second light period. The probable index for all three groups at this time was estimated by extrapolation of the control curve.

It can be seen from Figure 6 that the influences of the extracts of tritocerebral commissure and eyestalks are qualitatively the same as in the previous experiment. Tritocerebral commissure depresses while eyestalk supplements the degree of light-adaptation following equal light exposure. However, the rate of dark-adaptation of the animals receiving extract of tritocerebral commissure is substantially the same as for those receiving extracts of eyestalk, and, furthermore, this rate is much greater than that shown by the sea-water controls. Dark-adaptation of the two experimental groups was essentially complete in 270 minutes while the sea-water controls were still significantly light-adapted (distal pigment index of 0.068) at this time. It appears, therefore, that the tritocerebral commissure contains the dark-adapting principle but no significant amount of the light-adapting principle, while the eyestalks contain both principles.

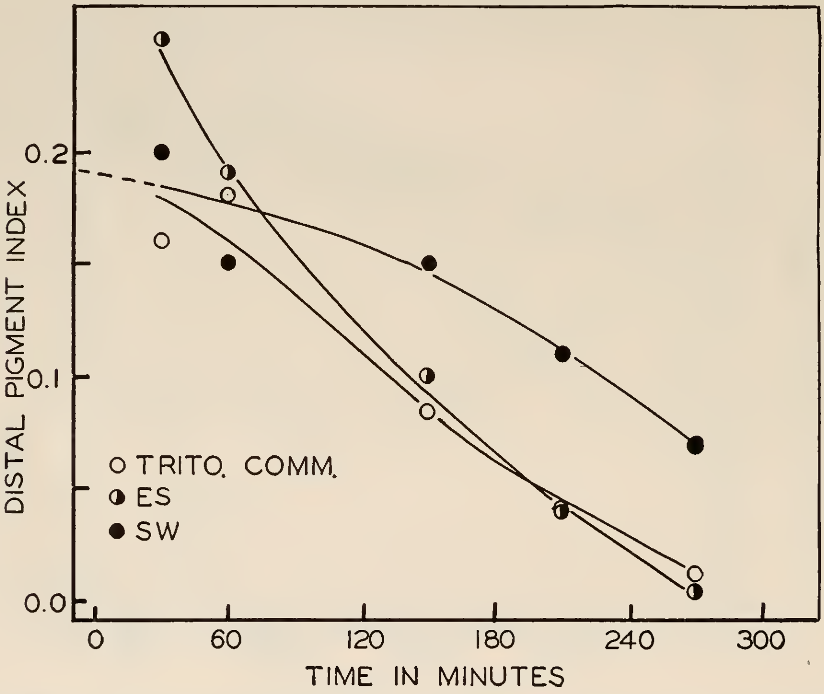

Figure 6. Comparison of the light- and re-dark-adaptational responses of prawns overnight in darkness to a simultaneous 250 ft.-c. light stimulus and injection of extracts or sea water. Trito. Comm.—tritocerebral commissure; ES—eyestalk; SW—sea water.

Discussion

The experiments involving the comparison of responses of animals with a single eye and eyestalk with those of normal animals clearly indicated that both eyestalks are essential to the normal regulation of the retinal pigment of a single eye. This phenomenon appears to be reasonably interpreted, if taken as an isolated observation, in terms either of (1) loss of one of the two major photoreceptors of the organism or (2) loss of an eyestalk source of an endocrine factor concerned in light-adaptation. In terms of the former, there would be expected to be a reduction in the number of afferent pathways activated by light and consequently of impulses passing into the central nervous system. There could conceivably, therefore, be a reduced excitation of any endocrine gland located anywhere in the body in response to any given intensity of illumination. Although this possibility is a real one, it is rendered less probable by the observation that removal of one eyestalk and eye does not significantly alter the rate of dark-adaptation of the remaining eye in response to a light-to-dark change.

The alternative interpretation, that the removal of one of the stalks has removed a major source of a light-adapting hormone, is equally likely, and this one has the added support that it is completely consistent with the view held for several years that the sinus glands of the eyestalks are the most important sources of a light-adapting hormone for the distal retinal pigment. This view is also given support by the experiments involving injection of extracts reported in this paper. The eyestalks are far more effective than any other organ of the body in light-adapting action.

By similar reasoning, using the failure of the removal of one eyestalk to alter the rate of dark-adaptation of the remaining eye, there is support for the view that the eyestalks are not, relatively, as important sources of a dark-adapting principle. This also is strengthened by the results of injection experiments.

The work reported here has substantiated earlier work (Brown, Fingerman and Hines, 1952) that following one complete discharge of light-adapting hormone, some hours are necessary before there is a regeneration of the capacity to respond as strongly again to an equivalent stimulus. Here it seems quite evident that the endocrine sources become recharged through synthesis or accumulation of the hormone and discharge it again only in response to appropriate stimulation. This conclusion appears quite secure in view of the fact that the light-adapting hormone exercises a dominance over the dark-adapting one. That is, when both are present in substantial titers, the light-adapting principle appears to exert an action which is only very slightly depressed by the presence of dark-adapting hormone. Only when the conditions are such that there is a reduction in the quantity of light-adapting hormone present can the dark-adapting hormone exert that action which is then a function of its concentration.

On the other hand, the interpretation of the increase over some hours in darkness of the capacity to re-dark-adapt following the light-adaptational response to a one-minute, 250 ft.-c. flash is more difficult because of the aforementioned dominance. There may be either (1) a recharging of the endocrine glands concerned with the production of the dark-adapting hormone which is later discharged in response to the light-to-dark change which terminates the light flash, or (2) there may have been a gradual increase in blood titer of a dark-adapting hormone having such properties that it disappears only slowly from the blood over a period of some hours.

There is still too little information to permit one to reach a decision as to whether the dark-adapting hormone becomes stored in the glands of its origin in darkness.

The results do suggest, however, that there is no accumulation of light-adapting hormone in the sources in animals which are maintained for some hours in constant light at 250 ft.-c. Such animals, immediately following dark-adaptation during which it is presumed there would be no induced liberation of light-adapting hormone, have but little power of responding to a brief light flash by light-adaptation. On the other hand, there is the suggestion that the sources of dark-adapting hormone are provided with accumulated dark-adapting hormone when they are taken from constant illumination or during the day from the laboratory tanks where they have been subjected to the normal daily variation of illumination. Such animals can dark-adapt rapidly. However, immediately following induced dark-adaptation, these animals are unable to respond to a light-to-dark change with other than a very slow rate of re-dark-adaptation of their distal retinal pigment.

As was done by earlier investigators, attempts were made during the course of this work to induce dark-adaptation of the distal retinal pigment in light-adapted animals maintained in light by injection of various extracts. It might be presumed from the work reported herein that extracts of tritocerebral commissures would have yielded such results, but this was not the case. There are two reasonable explanations for such failure. One of these is to be seen in the dominance of the light-adapting hormone and the fact that all of the experiments were performed at intensities of illumination producing complete or nearly complete light-adaptation. At such intensities the state of the pigment would be expected to be determined predominantly by the light-adapting hormone. A second possible explanation is that the regulatory powers of the animals to illumination are such that the animals compensate for any alterations due to the injections by the secretion of more of the dominant light-adapting principle. It is interesting, in this latter connection, that the only condition under which it was possible in this work to demonstrate the presence of a dark-adapting hormone by injection was under the environmental condition of complete darkness. This condition would be expected to call forth maximal reduction in the blood titer of light-adapting hormone, and furthermore this is a condition to which there could not be expected to be any active adjustment.

Finally, it is evident from the experiments which have been described that, at least in darkness, injected light-adapting hormone disappears more rapidly from the blood than does injected dark-adapting hormone.

SUMMARY

1. After removal of one eyestalk the distal pigment of the intact eye of *Palaemonetes* light-adapts more slowly and to a less extent for a given illumination; dark-adaptation is unaffected.

2. The eyestalks are the chief sources of light-adapting hormone; lesser amounts are found in brain, connectives and ventral ganglia.

3. The eyestalks and central nervous organs are sources of dark-adapting hormone. The tritocerebral commissure possesses dark-adapting, but no light-adapting hormone.

4. Both light- and dark-adapting hormones can be elaborated and stored, to be discharged in quantity upon appropriate stimulation.

5. Light-adapting hormone is elaborated and stored during a few hours in darkness; no store appears to be present in prawns kept in light.

6. Dark-adapting hormone appears to be stored in animals in light; following transfer to darkness, the store is depleted but the capacity to re-dark-adapt following response to a brief light flash gradually increases during a few hours in darkness.

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EFFECT OF ADRENAL PREPARATIONS ON TUMOR GROWTH

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Evidence has been accumulating in the literature for an influence of the adrenal cortex on malignant growth. Beck and Diller (1946) and Diller *et al.* (1948) reported definite degenerative changes and decrease in size of transplanted mouse sarcoma 37 following intraperitoneal injections of 0.5 to 1.0 cc. of a potent adrenal extract.

It seemed of interest to us, therefore, to investigate the effects on sarcoma 37 of more extended treatment with adrenal extract and with certain fractions obtainable from the adrenal. The effect of desoxycorticosterone acetate (DCA) was observed for comparison only since this substance is not an adrenal hormone.

METHODS

All animals used in this study were adult albino mice of the Carworth Farms strain, 10 to 12 weeks of age. A number of animals bearing sarcoma 37 were obtained from the Lankenau Hospital Research Institute through the courtesy of Dr. Irene Corey Diller. The tumor was kept growing in our own laboratory by transplanation into female mice every 10 to 14 days. This material provided the stock tumor for all experiments. Since sarcoma 37 sometimes shows spontaneous regression, experimental and control groups were always implanted and run simultaneously in the following manner, after the method of Dr. Diller (Diller, 1947). Viable tumors were removed from the host aseptically, divided into fragments and drawn into a number 15 trocar for implantation. Inoculations were made subcutaneously into the ventral surface of the recipient, the skin having previously been defurred and cleaned with 70 per cent alcohol. Transplants were routinely allowed a seven day development period. The tumor-bearing mice were then divided into three groups on the basis of tumor size as determined by palpation, *i.e.,* large, medium and small tumors. When animals for the experimental and control series were chosen, equal numbers were taken from each of these groups. In most cases, except as noted below, they were injected twice daily with the various preparations for periods ranging from 5 to 7 days. The age of the tumors at the time the animals were cancelled was 15–17 days. On the day following the injection period experimental and control groups were killed with ether. After being measured with calipers along two axes, one in a right angled plane to the other, the tumors were carefully removed and the living tissue separated from necrotic areas with the aid of a binocular loupe. The living tissue was weighed in a glass-stoppered weighing bottle either from individuals or from experimental groups. In some cases as noted, dry weight was also determined. The following preparations were used in experiments as designated: 1. Upjohn's whole adrenal extract, 1 cc. = 60 g. tissue; 2. Whole adrenal extract prepared in our own laboratory, 1 cc. = 60 g. tissue; 3. DCA[1] in oil, 1 cc. = 5 mg.; 3a. DAC in 15% alcohol, 1 cc. = 5 mg.; 4. sodium hormone in 10% alcohol prepared in our laboratory by Dr. J. S. Thatcher, 1 cc. = 300 g. tissue; 5. 17-hydroxy-11-dehydrocorticosterone[2] in 10% alcohol, 1 cc. = 0.6 mg.; 6. 11-dehydrocorticosterone[3] in 10% alcohol, 0.6 mg./cc.

RESULTS

Effect of whole adrenal extract

The results with adrenal extract, though variable, were so pronounced as to leave little doubt of its power to inhibit the growth of sarcoma 37 in female mice when adequate dosage was used (see Table I). Thus, one injection of 0.5 cc. (Group 5) five days before cancellation had no effect on tumor growth; 0.1 cc. injected daily for 5 days (Group 6) prior to cancellation caused a negligible (18 per cent) reduction in amount of viable tumor tissue present considering the variability, while 0.2 cc. injected daily for 5 days (Group 6) brought about almost complete disappearance of viable tissue as compared with controls. At the dosage level of 0.2 cc. daily for five days, there was a 28% reduction in amount of viable tumor tissue

TABLE I

Effect of whole adrenal extract on tumor growth

Group treatment	No. of mice	Dosage daily, cc.	Wt. of living tumor tissue				Degree of necrosis			
			Wet wt. avg.	% change	Dry wt. avg.	% change	0	+	++	+++
1 Adrenal Ext. A	12 ♀	.1 4×	.151	+104	.122	+198				
Controls	13 ♀		.069		.041					
2 Ext. A	15 ♀	.3 2×	.137	+37	.029	+27				
Controls	15 ♀		.100		.023					
3 Ext. B	17 ♀	.4 2×	.076	−42	.016	−41	0	5	6	6
Controls	20 ♀		.131		.027		1	7	8	1
4 Ext. B	9 ♂	.4 2×	.154	+208	.030	+123				
Controls	8 ♂		.050		.013					
5 Ext. C	7 ♀	*	.262	0			0	3	4	0
C	7 ♀	.1 1×	.213	−18			0	3	2	2
C	7 ♀	.2 1×	.031	−88			0	0	2	5
Controls	7 ♀		.260				0	5	2	0
6 Ext. C	11 ♂	.1 2×	.189	−28			2	3	4	2
Controls	10 ♂		.264				1	2	7	0

*—1 inj. only, 0.5 cc. 5 days before cancellation.
A—our own.
B—Upjohn, lot 1.
C—Upjohn, lot 2.
0—none.
+—slight.
++—moderate.
+++—complete.

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[1] Kindly supplied by Roche-Organon, Inc.

[2] Supplied through the courtesy of Dr. E. C. Kendall.

[3] Kindly supplied by Merck and Co.

in male mice (Group 6). This effect is remarkable since in males this tumor has been found notably resistant to adrenal preparations (Diller *et al.,* 1948). Another batch of adrenal extract was found to have no inhibiting effect at daily dosage levels of 0.4 and 0.6 cc., but at a level of 0.8 cc. daily the inhibition of sarcoma 37 in female mice was marked (Table I, Group 3), amounting to 42%. This extract at the same dosage level caused an actual increase in tumor growth in male mice. It is interesting to note that extract seems to reduce tumor size not so much by inhibiting growth as by accentuating the processes leading to necrosis. The data on degree of necrosis show this. In the group of 7 animals treated with 0.2 cc. extract C daily (Group 5), two showed a moderate degree of necrosis (++) while in five necrosis was complete (+++). In the controls of this series two exhibited a moderate degree of necrosis (++) while in five necrosis was very slight (+). Again in Group 3 about the same relationship obtains as to degree of necrosis observed, there being 6 out of 17 tumors completely necrotic (+++) in the extract-treated group while only one tumor out of 20 in the control series showed total necrosis. This observation is further substantiated by tumor measurements on this group at the time of killing. The average measurements on the control tumors were 91 × 127 mm. while those on the experimental group were 98 × 125 mm. or approximately identical. However, on weighing the living tissue from each group, the average value for the treated animals was found to be 0.076 g., 40 per cent below that of the control animals (0.131 g.).

Effect of sodium-retaining substances

Because of the small amount of sodium hormone available, this treatment was tried in only one group of 15 animals (Table II, Group 5). It resulted in a questionably significant reduction (26 per cent) in living tumor tissue when compared to the control group. As in the case of whole adrenal extract the reduction appeared to be due to more rapid progress of necrosis rather than to direct

TABLE II

Effect of sodium-retaining substances on tumor growth

Group treatment	No. of mice	Dosage daily, cc.	Wt. of living tumor tissue				Degree of necrosis			
			Wet wt. avg.	% change	Dry wt. avg.	% change	0	+	++	+++
1 DCA in oil	6 ♀	*	.172	+60			0	2	2	2
Controls	6 ♀		.108				0	1	1	4
2 DCA in oil	13 ♀	.1 4×	.243	+254			10	3	0	0
Controls	13 ♀		.069				9	3	1	0
3 DCA in alcohol	15 ♀	.3 2×	.175	+75	.036	+55	2	6	4	1
Controls	15 ♀		.100		.023		5	7	2	1
4 DCA in oil	11 ♂	.1 2×	.312	+18			2	5	4	0
DCA in alcohol	11 ♂	.1 2×	.272	+3			0	4	6	1
Controls	10 ♂		.264				1	2	7	0
5 Sodium factor	15 ♀	.1 2×	.085	−26	.018	−22	1	4	7	3
Controls	15 ♀		.115		.023		9	3	3	0

* 0.5 cc. subcut., 0.5 cc. intraper. 7 days before cancellation.

TABLE III

Effect of some crystalline gluconeogenic substances on tumor growth

Treatment	No. of mice	Dosage	Wt. of living tumor tissue	
			Wet avg.	% change
17-hydroxy-11-dehydrocorticosterone (cortisone)	7 ♀	1 inj. 300 γ in 10% alc. 5 days before cancellation	.428	+65
11-dehydrocorticosterone	7 ♀	1 inj. 300 γ in 10% alc. 5 days before cancellation	.385	+48
Controls	7 ♀		.260	

inhibition of growth. It will be noted in the table that three sodium hormone-treated animals exhibited complete necrosis (+++) while seven showed a moderate amount (++) and only one animal showed none, while in nine animals of the control group no necrosis was observed and none of the others showed marked necrosis. This rather striking difference in necrosis in favor of the sodium hormone-treated animals further strengthens the questionably significant data on tumor tissue weights. The synthetic sodium-retaining substance, DCA, had the opposite effect on tumor growth. All dosages employed produced marked increases in tumor growth in female animals; in the male insignificant increases were observed (Group 4, Table II). DCA in oil appeared to be a more potent tumor growth stimulant (254 per cent increase) than DCA in alcohol (75 per cent increase), probably because in alcohol the substance is more rapidly absorbed and destroyed. It will be noted (Group 3) that the increase in living tumor tissue is significantly more on the wet weight basis than on that of dry weight. This is to be expected from the known action of desoxycorticosterone acetate in increasing body water generally.

Effect of crystalline gluconeogenic substances

The amounts of crystalline gluconeogenic compounds available at the time these observations were made seriously limited our study of these substances. Seven female mice were injected subcutaneously with 300 gamma each of 17-hydroxy-11-dehydrocorticosterone (cortisone) and an equal number of mice with the same amounts of 11-dehydrocorticosterone. All were cancelled five days later. Both substances definitely increased tumor growth as compared to controls (Table III), and there was no significant difference between them. In the case of the cortisone-treated animals there seemed to be some increase in amount of necrosis over that found in control animals. This might be due to the cortisone treatment or possibly to larger tumors more rapidly outgrowing their blood supply. From the data available there is no means of differentiating these two factors.

DISCUSSION

There is some evidence that a low level of adrenal cortical secretion is associated with tumor growth and that a high level is inhibitory to such growth. Thus, Haven and Asheworth (1950) found steroids in the adrenal markedly decreased in rats bearing Walker tumor 256 even though the gland weights in these animals were significantly increased, and Dobriner and co-workers (1950) found urinary ketosteroid excretion low in patients with neoplastic disease. Haven *et al.* (1949) observed somewhat increased steroid content of the adrenals of rats in which Walker tumor 256 either did not take, or in which it took and regressed.

Of the direct, inhibitory effect of adrenal grafts, adrenal tissue extracts, synthetic adrenal hormones or synthetic products possessing some properties similar to adrenal hormones, most observations seem to be on lymphoid tumors, or leukemia (Law and Speirs, 1947; Murphy and Sturm, 1944, 1950; Woolley, 1950). These authors all observed at least temporary regression. Their results are in line with the well known observations of involution of the thymus and lymph nodes when additional cortical hormones are administered to normal animals. Of such effects on sarcoma 37, the only evidence of which we are aware aside from our own (Table I) is that of Beck and Diller (1946) and Diller *et al.* (1948) using small amounts (0.5 to 1.5 cc.) of Upjohn's adrenal extract as noted in the introduction. In our hands one injection of 0.5 cc. Upjohn's extract had no effect on growth (Table I, Group 5) as measured by weight of viable tissue five days after the treatment. There are two possible reasons for this difference. First, we were using a different batch of extract and second, we cancelled our animals five days after the injection while Diller *et al.* (1948) noted the greatest incidence of tumor disappearance 17–35 days after treatment.

Our results using cortisone (Table III) are at variance with those reported in the literature in that with sarcoma 37 it produced an actual increase rather than decrease in tumor growth. Heilman and Kendall (1944) working with lymphoid tumors, Higgins *et al.* (1950) using transplanted rhabdomyosarcoma and Burchenal *et al.* (1950) observing leukemic mice obtained results in the direction of inhibition or regression. All of these workers used massive doses of cortisone.

Both large and small doses of DCA (Table II) also stimulated the growth of sarcoma 37. The work of Lipschütz and Zañartu (1942) showed this substance to be anti-fibromatogenic in female guinea pigs treated with estrogens. On the other hand, Kupperman and Greenblatt (1946), working with transplanted sarcoma in rats, obtained results comparable to ours (Table II), namely an enhanced growth of the tumor in animals treated with DCA. It will be noted (Table II, Group 3) that the increase in tumor tissue is considerably greater on the wet (75 per cent) than on the dry weight (55 per cent) basis. This is in line with observations in the literature on other tissues taken from animals treated with DCA (Zuckerman *et al.*, 1950). Although the sodium hormone acts like DCA in causing sodium retention it is unlike this substance in other respects (Thatcher and Hartman, 1946, Hartman *et al.*, 1939). It is interesting to note that the two compounds also differ in their effect on sarcoma 37; DCA caused a marked increase in growth of the tumor while sodium hormone caused a questionably significant reduction in amount of viable tissue present at autopsy and more rapid progress of necrosis than occurred in controls. The sodium hormone used on these animals was not a pure substance. It was purified only to the extent that it contained no gluconeogenic hormone and therefore presumably no compounds with oxygen in the C_{11} position. It is noteworthy that such an adrenal fraction possesses some power to inhibit the growth of sarcoma 37 and to promote necrosis. There is, in the finding of Dobriner *et al.* (1950), that steroids with oxygen in the C_{11} position are *not* decreased in the urine of patients with neoplastic disease while other steroids are, a further suggestion that other adrenal hormones are more important in this condition than those with gluconeogenic properties. Further investigation of the sodium hormone fraction is planned.

Summary

1. The effects of adrenal extract, sodium hormone, DCA, 17-hydroxy-11-dehydrocorticosterone (cortisone) and 11-dehydrocorticosterone on transplantable mouse sarcoma 37 have been investigated. More than 250 animals were used.

2. Injection of adrenal extract for 5 days caused as high as 88% decrease in viable tissue and marked necrosis of the tumor as compared to controls.

3. Sodium hormone caused a questionably significant decrease in viable tissue (26%) and marked increase in necrosis.

4. DCA increased tumor growth in all doses employed.

5. Both cortisone and 11-dehydrocorticosterone in single relatively small doses (300 γ) enhanced the growth of sarcoma 37.

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THE LARVAL DEVELOPMENT AND ECOLOGY OF THORACOPHELIA MUCRONATA (TREADWELL)

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The Opheliid polychaetes are well known for their localized distribution. Most species are restricted to soils with a relatively narrow range of particle size, some characteristic of fine muds, others of relatively coarse sands. *Thoracophelia mucronata,* like the common species of Ophelia, is found in sands subjected to a relatively heavy wave action, occurring inter-tidally in beaches experiencing fairly heavy surf on the Pacific coast of North America, from Vancouver Island (49° N) to the Punta Banda region, Mexico (31°30′ N). The ecology and life history of such species are particularly interesting, since the larvae, which in all known instances are planktonic, settle on a relatively restricted and unstable substratum.

The development of only one species of Opheliid, *Ophelia bicornis* Savigny, has been worked out in any detail (Wilson, 1948), and although the larval development of *Thoracophelia mucronata* follows a similar plan, there are a number of interesting differences. McConnaughy and Fox (1949) give a brief account of the early development of *Thoracophelia mucronata.* Feeding, and certain aspects of the biochemistry of metabolism, have been studied in this species by Fox, Crane and McConnaughy (1948), and McConnaughy and Fox (1949).

The present study was made over a period of rather less than one year from October, 1950, on the same population studied by Fox and his co-workers at La Jolla, California (32°52′03″ N, 117°15′11″ W).

I am glad of this opportunity to thank Professor Martin W. Johnson, and Professor Dennis L. Fox, of the Scripps Institution of Oceanography, University of California, for many courtesies.

Reproduction and Development

The species is dioecious, males and females occurring in approximately equal numbers. Externally alike, they can be distinguished only by the character of the coelomic germ cells. Coelomic oocytes are colorless, biconvex discs 65 μ in diameter, and 25 μ — 30 μ thick at the center (Fig. 1a, a′). The ripe spermatozoon (Fig. 1b) has a dark acrosome, and a tail about 45 μ in length. As in other polychaetes, the spermatozoa arise from "sperm plates" in the coelom. The worms are apparently atoquous, spawning taking place in or on the surface of the sand.

Fertilizations were easily achieved with really ripe gametes, by slitting open the adults and mixing the eggs and sperm in shallow dishes. Surplus sperm could be washed off, and the larvae which eventually swam to the surface transferred to larger vessels.

Development was extremely rapid (at 15° C. — 18° C.), most of the eggs having reached the 16-cell stage within 2 hours after fertilization. Polocytes are large and clearly distinguishable during the early stages of cleavage (Fig. 1c, e, f). The blastomeres of the 4-cell stage are approximately equal in size, so that the direction of rotation of the subsequent cleavage could not be determined merely by inspection. After about six hours an equatorial prototroch has developed, and the young larva swims off the bottom soon afterwards (Fig. 1g). An apical tuft of filaments and a single filament slightly dorsal to the vegetal pole are also distinguishable in the swimming trochophore (Fig. 1g). The larvae at this stage are positively phototactic, and are vigorous swimmers. After about five days the larva (Fig. 2a, a′) has reached a length of about 100 μ, and has become distinctly asymmetrical. The prototroch is more prominent, is composed of several rows of cilia, and completely encircles the larva. By this time a telotroch has also appeared, and the mouth and anus have opened into the gut. The anterior apical tuft and the posterior filament remain unchanged until they are lost at metamorphosis.

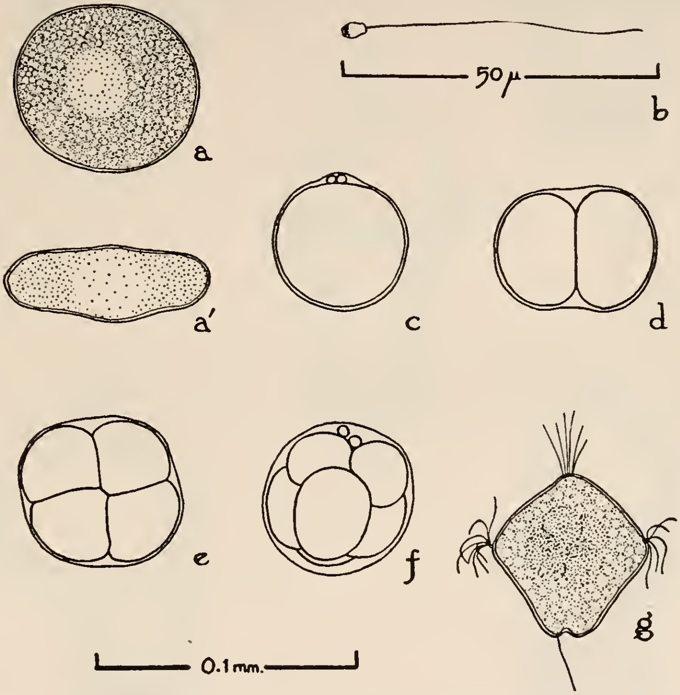

FIGURE 1. Early development. (a), (a′) ripe coelomic oocytes in surface and edge view; (b) spermatozoon; (c) fertilized egg with 2 polocytes; (d) 2-cell stage; (e) 4-cell stage; (f) 8-cell stage; (g) trochophore.

Throughout the remainder of its planktonic life the larva is markedly asymmetrical, the head region projecting ventrally, forming a large overhanging ciliated lip so that the mouth eventually comes to be directed posteriad, and the stomodeum and the anterior part of the gut are twisted so that the buccal cavity lies slightly to the right, the precursor of the proboscis to the left of the mid-line. Thus at this stage, and in older planktonic larvae, three distinct regions of the gut may be identified:

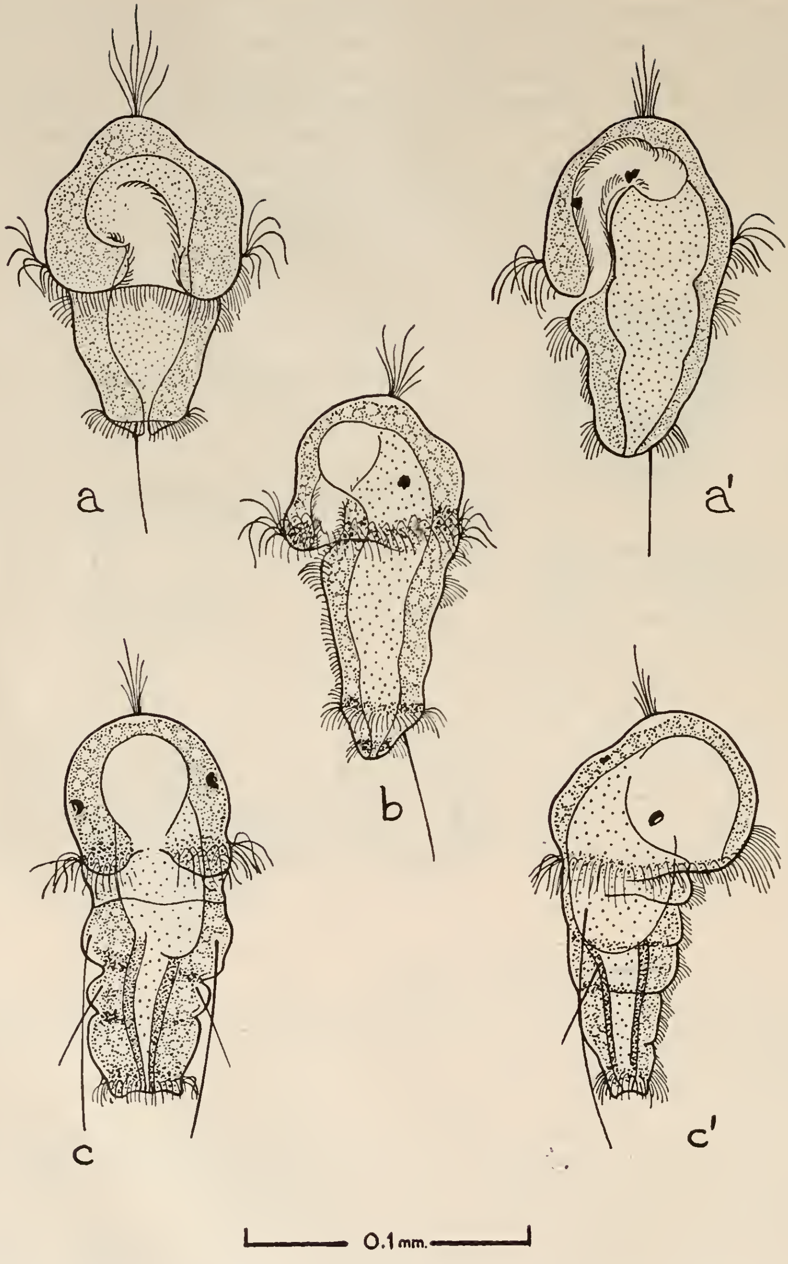

FIGURE 2. Larvae. (a), (a′) 5 days old, ventral view, and view from the left side; (b) 7 days old, view from the left side; (c), (c′) 9 days old, ventral view, and view from the right side.

(1) a ciliated channel opening into (2) a large non-glandular sac, the precursor of the eversible proboscis, and (3) a straight glandular part leading to the anus. Because of the rapid growth of the anterior part of the gut, it becomes not only bent and slightly twisted, but pushed forwards into the prostomium. This condition is apparently only temporary, since young bottom stages (Fig. 3a) show a relatively straight tube leading from mouth to anus.

As will be seen from Figures 2a′, b, c′, additional tufts or short bands of cilia arise from the ventral region of the larva, and in many larvae form a virtually continuous midventral ciliary band. One pair of rather irregular eye-spots has appeared by the fifth day after fertilization, and in later larvae, three or even four eyes may be seen. By the ninth day two chaetigerous segments have been formed, each at first bearing a single chaeta on either side (Fig. 2c, c′). The two pairs of chaetae appear in rapid succession, the anterior pair projecting well beyond the tip of the pygidium when the chaetae are held against the body while swimming. The first chaetae always appear to be simple capillaries, but winged bristles (Fig. 3b) commonly occur in later larvae. When the larva is nine or ten days old, transverse folds in the cuticle appear between the segments. A very definite division appears between the mouth and prototroch, and the first chaetigerous segment. This region just anterior to this groove may represent the first true segment; this problem is discussed below.

By the tenth day slight projections from the corners of the pygidium may be seen, apparently related to the cement glands which by their secretions enable the larva to adhere strongly to the substratum. Although attempts to rear larvae beyond this stage have not been successful, this almost certainly represents the stage of development at which the larva settles out of the plankton and becomes bottom-living. The apical tuft of filaments and the terminal filament are lost, while the pygidial cement glands aid the larva to maintain its position on a suitable substratum when once found. The planktonic life of these larvae is, therefore, probably not more than about ten days. The head is distinctly darker than the rest of the body at this stage. The trilobed proboscis characteristic of the adult is already differentiated in a larva with ten chaetigerous segments, and is eversible by the time the larva has attained a length of 1.0 mm. At this stage the prostomium is still rounded (Fig. 3a) and similar to other Opheliid larvae which have been described, and it is not until the larva is about 2.0 mm. in length that the prostomium has become pointed, and has coalesced with the first two chaetigerous segments (Fig. 4). These segments become cut off from the thoracic region by a transverse septum, the septa between the succeeding segments being absent or ill defined. The sac which opens dorsally through this transverse septum into the anterior or "head" coelom and which extends back to the fifth or sixth chaetigerous segment is already well defined in a young worm 2.5 mm. in length. The whole apparatus is probably an adaptation for burrowing, as McConnaughy and Fox suggest.

The four anal papillae associated with the cement glands (Fig. 3a) are posterior and ventral to the anus. None of these papillae represents the single large ventral cirrus of the adult (as McConnaughy and Fox have stated), since in young worms between two and four millimeters in length the four larval papillae may clearly be seen on the tip of the adult cirrus as it grows out from the pygidium (Fig. 3d). The dorsal cirri are similarly derived from the pygidium, although the last few

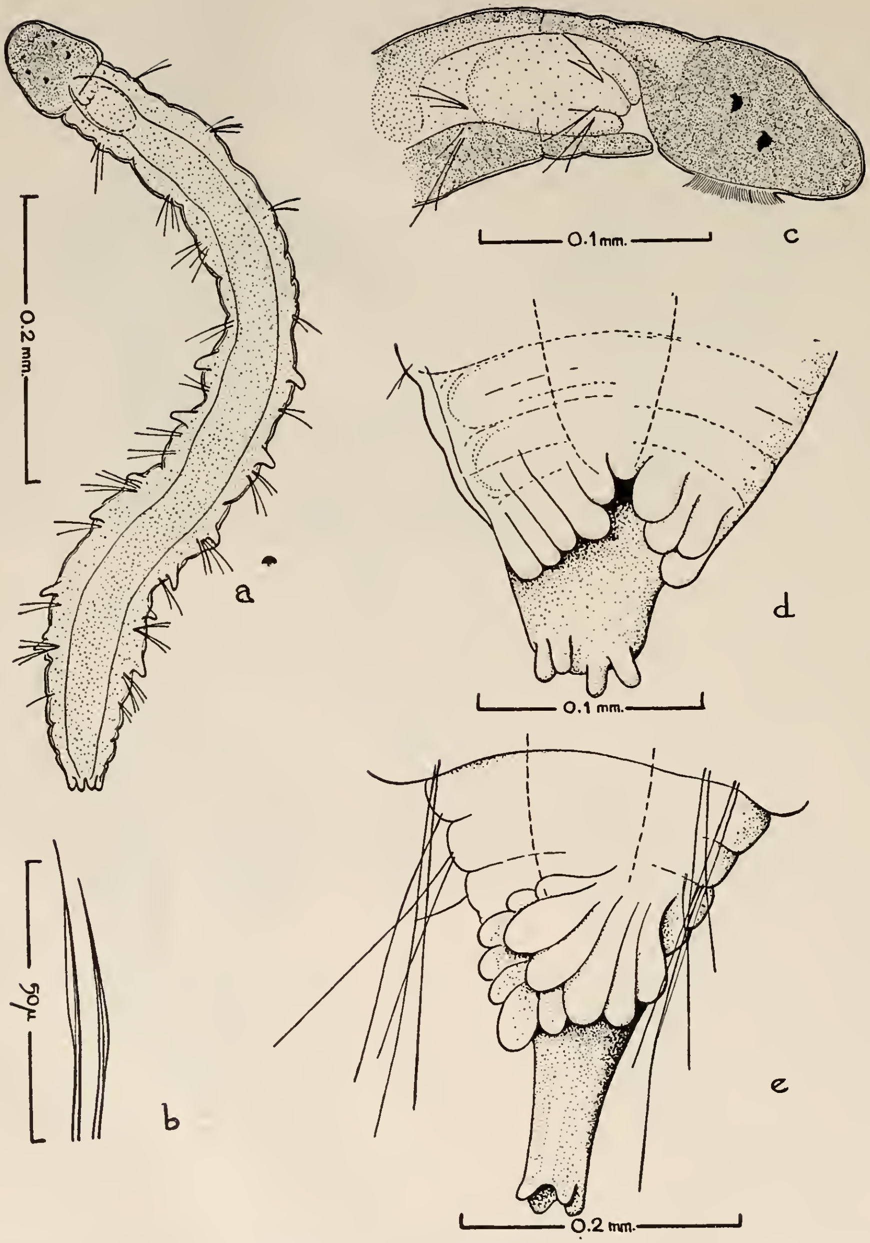

FIGURE 3. (a) Young bottom stage; (b) winged chaetae; (c) side view of the head region of a bottom larva 0.6 mm. long; development of the pygidial region; (d) condition in a young worm with about 26 chaetigerous segments; (e) in a young worm with 36 chaetigerous segments (4 mm. long).

chaetigerous segments become closely associated with it, the chaetae of these segments eventually projecting beyond the anus (Fig. 3e).

DISCUSSION

It is interesting to compare the larval development of *Thoracophelia mucronata* with that of *Ophelia bicornis* as described by Wilson:

Ophelia bicornis	*Thoracophelia mucronata*
Ripe females metallic green in color; males white or pale cream	Ripe males and females similar, the adults containing so much hemoglobin that sexually mature individuals appear only slightly whiter than immature or recently spawned worms
Mature coelomic oocytes oval plates 150 μ × 130 μ	Mature coelomic oocytes biconvex discs 65 μ × 25 μ
Trochophores produced within 24 hours	Trochophores produced within 6 hours

Common features:

Trochophore rounded or slightly conical with an apical tuft, and a single filament projecting from the vegetal pole.

Larva at the time of settling with 2–3 chaetigerous segments; the head rather darker than the rest of the body, and the pygidium with adhesive papillae. Eyespots rather irregular in shape and number. Winged chaetae present.

The eggs are distinctly smaller than those of *Ophelia bicornis* described by Wilson, and this, together with the higher temperatures under which the *Thoracophelia mucronata* normally develops, accounts for the more rapid development in the early stages. Even so, the larvae of *Thoracophelia mucronata* do show a certain acceleration in the development of some structures as compared with *Ophelia bicornis.* The structure of the adult *Thoracophelia* is more specialized than *Ophelia* in the possession of an anterior "head coelom" in relation to burrowing, separated as its name implies from an aseptate "thoracic" region. The proboscis is somewhat precocious in its development, the anterior region of the gut becoming pushed forwards and coiled almost within the prostomial region as already described. Owing to surf action on the shores on which the larvae settle, the early development of burrowing structures would probably be of advantage. The shape of the curve in Figure 5 may also be interpreted as a tendency for a relatively rapid delimitation of segments early in development. It will be seen (Fig. 5) that a *Thoracophelia mucronata* larva has twenty-five chaetigerous segments when 2.0 mm. in length, while *Ophelia bicornis* larvae do not possess this number of segments until they have reached a length of 5.0 mm. By the time young *Thoracophelia mucronata* have reached a length of 5.0 mm. the number of segments usually found in adults (38) has been attained, although old worms may be ten times this length.

Wilson describes in *Ophelia bicornis* the fusion of the first segment with the prostomium, though remaining separated by a groove. The existence of this segment is not clear in *Thoracophelia mucronata,* and is certainly not recognizable in later larvae (Fig. 3a). However, in larvae with two chaetigerous segments there is a clear demarcation between the mouth opening and the first chaetigerous segment (Fig. 2c). It is suggested that this region between the mouth and the first chaetigerous segment represents the first true segment, which in *Ophelia bicornis* is achaetous but distinct, but which in *Thoracophelia mucronata* becomes almost obliterated. That this is likely is shown by the fact that the foregut does develop so far forward as already described, and that the precursor of the eversible proboscis does at first lie anterior to the first chaetigerous segment. Since in most polychaetes the proboscis arises almost entirely within the first segment, it is reasonable to assume that the first chaetigerous segment of *Thoracophelia mucronata* represents the

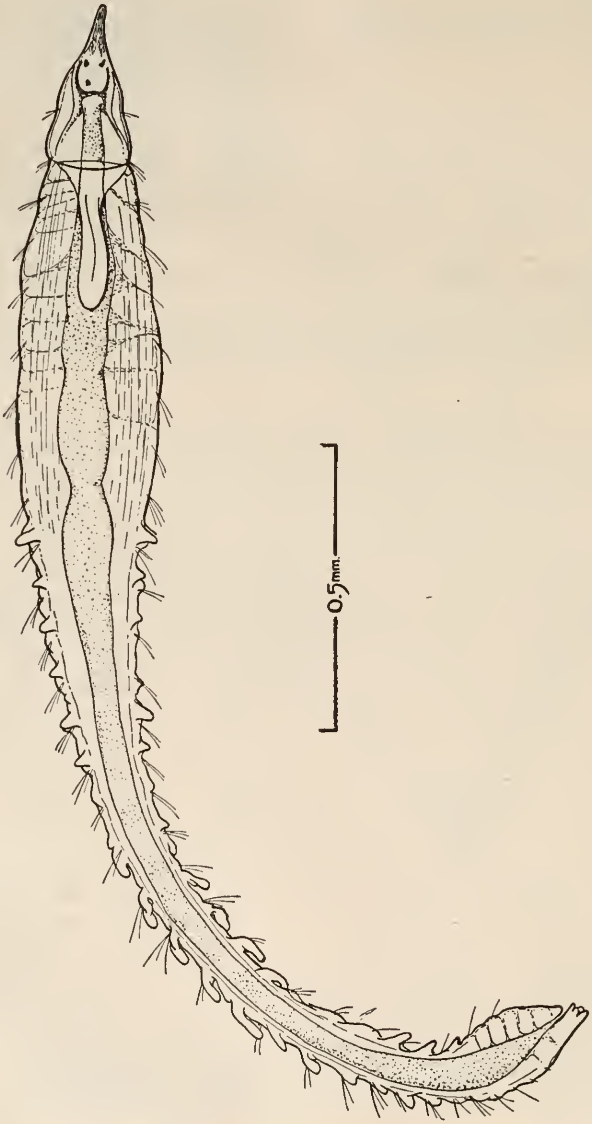

FIGURE 4. Young *Thoracophelia mucronata*

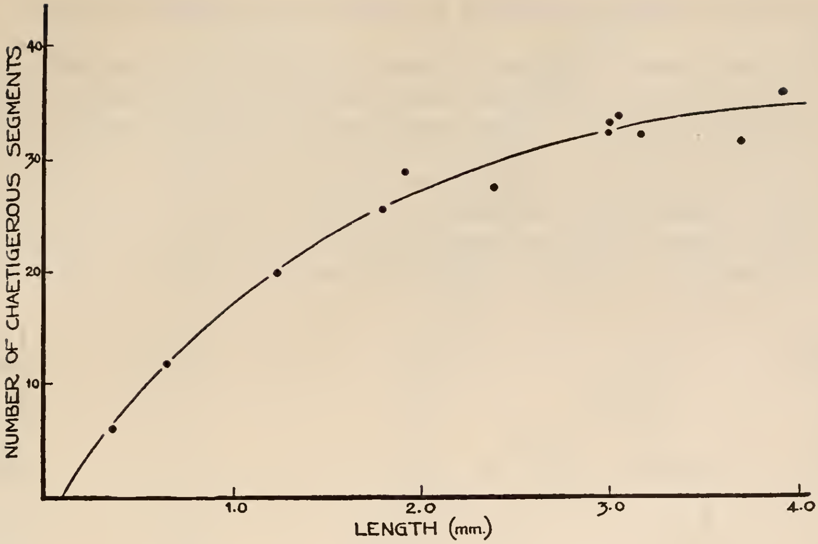

FIGURE 5. Relation of length to number of chaetigerous segments.

second segment, and is homologous with the first chaetigerous segment of *Ophelia bicornis*.

ECOLOGY

The breeding period of *Thoracophelia mucronata* is prolonged during the summer, probably owing indirectly to the equable and relatively high temperatures experienced throughout the year (the temperature of the surface water at high tides varying from a summer maximum of about 22° C. to a winter minimum of about 13° C.). Consequently, the mean length of the worms comprising the population did not vary appreciably from month to month.

Another factor to be considered is the stirring of the surface sand by the surf. Large worms tend to burrow down under such conditions (McConnaughy and Fox, 1949), but a certain proportion are moved after each tide. After rough seas whole areas of the shore normally heavily populated will be depleted of individuals which will be found in immense numbers in channels and pockets where the current loses speed or changes direction. Thus, in spite of the tendency of the larger worms to burrow down, there is an efficient shuffling of the population from time to time, and it is difficult to decide how far the distribution of the worms in the beach is due to purely mechanical agencies. The large amount of hemoglobin which they contain probably acts as an oxygen-store during the periods when the worms burrow deeper to avoid being swept away. When uncovered by the tide, the worms burrow upwards, a small hole appearing on the surface above each worm. This habit is probably respiratory in function, since the worms are usually found head downwards under such conditions, the rectum being used as a kind of lung. The great development of sensory papillae around the anus may thus be understood. The elongate chaetae of the most posterior segments may play some part in breaking the surface and preventing the sand from falling in around the anus while respiration is in progress.

The general pattern of zonation is shown in Figure 6. The six stations were spaced at intervals of about 6 meters between high and low water marks. Samples were taken each month using a square frame with sides 25 cm. in length (enclosing an area of 625 cm.^{2} or $\frac{1}{16}$ m.^{2}), the sample being passed through a sieve with round holes 0.5 mm. in diameter. It will be seen (Fig. 6) that worms extend from low water mark to almost high water mark of ordinary tides, and are not restricted to a relatively narrow belt as McConnaughy and Fox have stated. This belief was due to the restriction of the larger worms to the upper part of the shore, the population being most dense just above mid-tide level, while the younger worms—which are usually overlooked in the sand owing to their transparency—are restricted almost entirely to the lower part of the shore. A clear division is apparent between the size distribution of worms taken above the mid-tide level where a certain degree of drying out takes place at low tide, and below mid-tide level where the sand remains at or near saturation point (Fig. 7). Two explanations of this division are possible. The larvae may settle over the whole intertidal zone but those above mid-tide level either do not survive owing to the rise in temperature or drying out of the surface sand, or are carried down the beach by the surf. The larger worms are not washed out because of their greater size and weight and ability to burrow be-

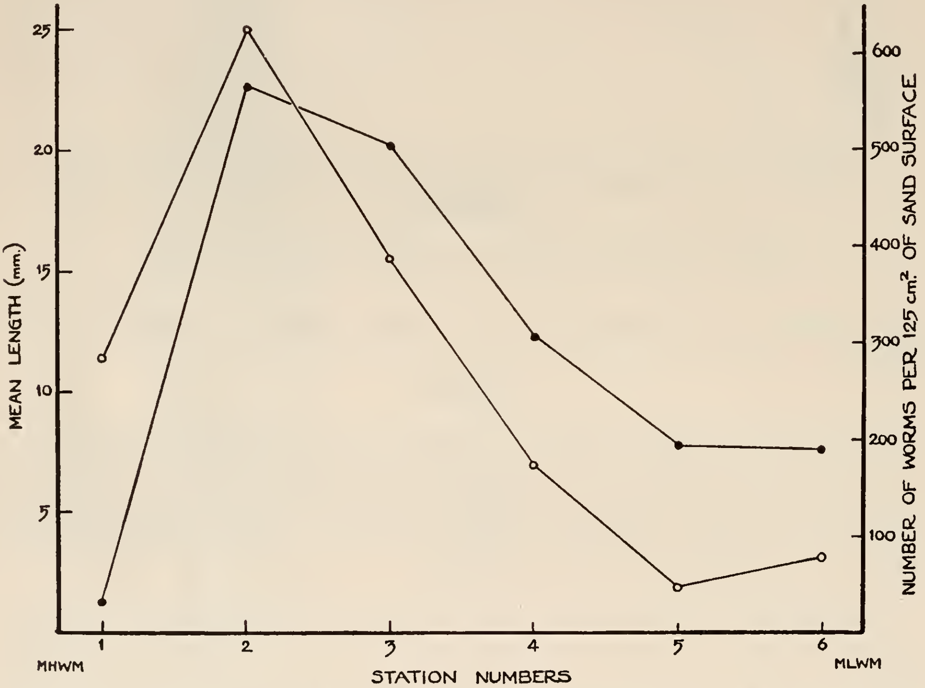

FIGURE 6. Zonation of *Thoracophelia mucronata* at La Jolla. Filled circles = number; empty circles = length.

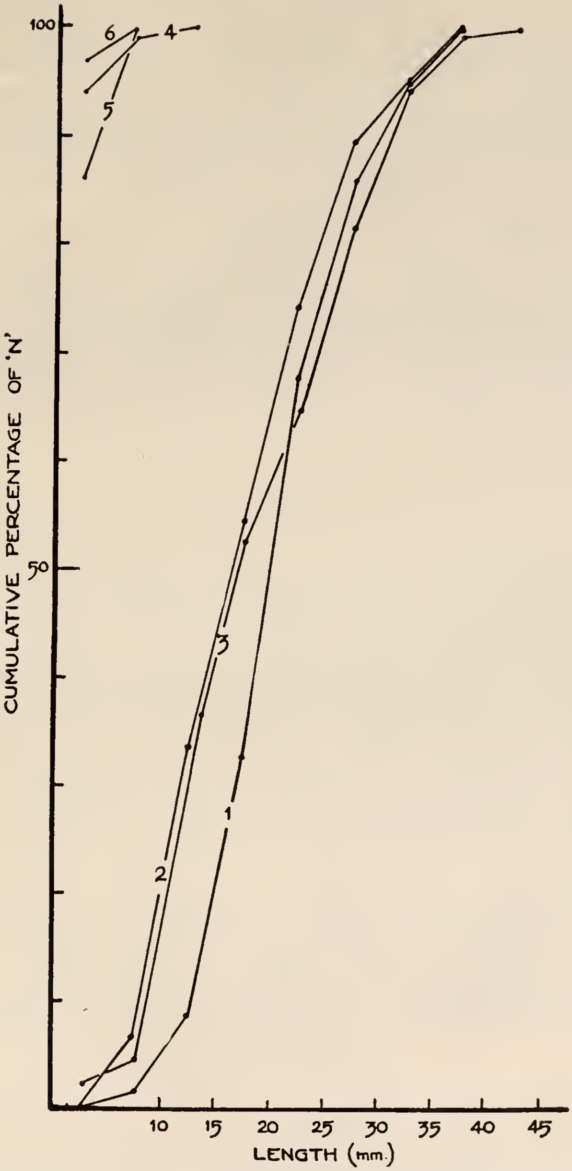

FIGURE 7. Size-distribution of worms at six stations from just below high water mark (1) to just above low water mark (6). Station numbers as in Figure 6.

neath the stirred layer. Secondly, the larvae may settle only below mid-tide level and later migrate upshore. However, it is clear that the optimal zone for the adult worms is appreciably higher than that for the younger stages, but the reason for the relatively sharp division in the mean length of worms from the two zones is not known.

Although there is no doubt that large worms may be redistributed by wave action, under normal conditions there is a certain degree of aggregation as in most polychaetes. The mean density of population at the optimal level was about 10,000–11,000/m.², but within an area of two or three square meters 625 cm.² samples might contain between 500 and 900 individuals (8000–14,000/m.², approximately). Since fully grown worms weigh about 0.04 gm. (McConnaughy and Fox, 1949) and are continually passing sand through the gut, their effect on the mechanical structure of beaches where they occur may be appreciable.

Owing to the large amount of hemoglobin which these worms possess, their food-value for other organisms is considerable, and they constitute an important item in the diet of many shore birds, especially the marbled godwit *Limosa fedoa*.

From a study of the figures obtained from the samples made throughout the year, and from observations made under laboratory conditions, it seems likely that these worms take more than one year to mature, and it is probable that each individual lives for a few years, possibly spawning several times.

SUMMARY

1. The larval development of *Thoracophelia mucronata* follows the same pattern as that of *Ophelia bicornis,* but is much more rapid, the eggs being less than a quarter of the volume of those of *O. bicornis* when mature.

2. The larval development shows differences in the formation of the anterior segments and the anterior regions of the gut.

3. Larvae of both species show the development of the adhesive papillae at the time of settling, and this is regarded as an adaption to settling on an unstable substratum.

4. The great development of hemoglobin in *Thoracophelia mucronata* may be interpreted as an adaptation against surf action, the hemoglobin functioning as an oxygen-store when the stirring of the surface layers of sand causes the worms to burrow more deeply.

5. Attention is drawn to the development of sensory papillae, and the elongated chaetae of the most posterior segments in relation to rectal respiration which is carried on near the surface when the sand is not being disturbed.

6. The optimal tidal level for adult worms is above that of the younger stages; the relation between zonation and the size of the worms is discussed.

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IRON ASSIMILATION BY MARINE DIATOMS[1]

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The relationship of marine productivity to the availability of iron has been discussed by Gran (1933), who suggested that organically combined iron introduced into coastal waters from terrestrial run-off is responsible for phytoplankton abundances. He substantiated his argument by demonstrating that the growth of the neritic diatom *Skeletonema costatum* was stimulated by soil extract containing 0.2 micromole of iron per liter, although the iron bound into oxyhemoglobin molecules had no effect. Hopkins (1930) has indicated that ionic iron is necessary for the growth of *Chlorella,* and Rodhe (1948) defines the available iron for utilization in culture by fresh water algae as that which has been stabilized by combination with citrate.

Harvey (1937a) has demonstrated that diatoms are able to assimilate ferric hydroxide or ferric phosphate in colloidal or particulate form and to utilize it in their growth. He has further shown, using the calculated saturation concentrations of ferric and ferrous ions in marine waters, that diatoms accumulate 10,000 times more iron than could possibly be obtained from diffusion of iron ions from the environmental waters. His treatment considered a stationary spherical living cell with respect to the environment, whereas a falling diatom would encounter and take up somewhat more iron.

Continuing an investigation of the minimal quantities of mineral nutrients needed for optimal growth of the marine diatom *Asterionella japonica* (Goldberg, Walker and Whisenand, 1951), an assessment of iron uptake presents the following questions. What is the minimal content of iron per cell needed for further division? What constitutes available iron? Finally, inasmuch as the concentration of iron in marine waters has been found to be highly variable (Cooper, 1948), can iron content be one of many possible parameters in the nutrient index of the productivity equation of Riley, Stommel and Bumpus (1949)?

CULTURE TECHNIQUES

The culture techniques used in the following experiments, unless otherwise indicated, have been described previously in the work on phosphate uptake (Goldberg, Walker and Whisenand, 1951). To maintain symmetry with respect to these investigations the same culture of *Asterionella japonica,* which was isolated from the plankton samples in the winter of 1950, was used. The initial phosphate level was adjusted to 2.5 to 3.0 micromoles of phosphate per liter.

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[1] Contribution of the Scripps Institution of Oceanography, New Series No. 572.

IRON ASSAY

The iron determinations were made either by the colorimetric method of Buch (1942) or through the use of radioactive iron. In the former procedure the extinction of the alpha-alpha' bipyridyl complex of iron was measured at 522 mμ in 100 mm. cells in a Beckman Model DU Spectrophotometer. The method determines both ferric and ferrous iron, but only a portion of the organically bound or particulate iron.

Iron [2] containing the radioactive isotopes Fe^{55}, which decays by K capture, and Fe^{59}, a beta and gamma emitter, was employed as a tracer for this element. The radio iron was plated from aliquots of sea water samples onto 10 cm.^{2} copper planchets following the procedure of Ross and Chapin (1942). As assayed under an end-window Geiger-Muller tube with a window thickness of 1.8 mg./cm.^{2}, the specific activity was 2500 counts per minute per micromole.

IRON PARTITION IN CULTURE BOTTLES

In the first experiments with radioactive iron it was found that 80 to 90 per cent of the iron added initially as the ferric citrate complex was adsorbed within one day to the walls of the culture bottle. To eliminate this adsorption, the culture bottles were coated with "Desicote," a polymer of silicon (manufactured by Beckman Instruments, Inc., South Pasadena, California). The coated bottles had no adverse effect upon diatom growth, as was evident from control runs in both treated and untreated bottles. The bottles retained their coating for periods up to one month. At the end of an experiment the bottles were washed with a detergent and thoroughly rinsed with distilled water.

Radioactive ferric citrate, freshly prepared, was introduced into sea water of pH 8.00 to give an iron concentration of 2.2 micromoles per liter. Aliquots of 10 ml. of the solution were centrifuged for thirty minutes and the supernatant liquid assayed radiometrically for iron. The results are listed in Table I. It is observed that hydrolysis of iron citrate occurs to a considerable extent in a period of four days.

As is noted in the table, the decomposition was noticeably accelerated both by light and by stirring. The light effect is probably similar to that reported by Peltz and Lynn (1938) who found that iron citrate complex was partially decomposed in sunlight to CO_2 and a soluble ferrous ion. The ferrous ion formed in this way can readily be oxidized to give the ferric hydroxide. The more pronounced effect of stirring upon the hydrolysis is undoubtedly due to the formation of large particulate masses by collisions of smaller iron hydroxide particles, and to more rapid oxidation of any ferrous ion formed.

As pointed out by Harvey (1945), iron ascorbate has less tendency to become hydrolyzed in saline media, if the solution is freshly prepared. On standing, stock solutions of iron ascorbate, originally tinted but a very light green, assume deep brown colorations. It was found that ferric ascorbate was not decomposed for periods up to one week, but after longer periods the formation of centrifugable iron hydroxide followed the course of iron citrate solutions. Iodoform tests on both the

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[2] The radioactive iron metal used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

Table I

The hydrolysis of iron complexes in sea water solutions

Complex of ferric ion	Time elapsed since addition of iron complex (days)	% of original iron remaining in supernate	Remarks
Citrate	0	100	Light, stirred
	1	64	
	2	29	
	3	20	
	4	11	
	6	6	
Citrate	4	50	Dark, unstirred
	4	10	Light, stirred
	4	31	Light, unstirred
Ethylene diamine tetra-acetate	0	100	Light, stirred
	1	88	
	3	80	
	6	15	

ferric citrate and ferric ascorbate solutions were positive within two weeks after preparation.

Ethylenediamine tetra-acetic acid chelates with trivalent iron and this complex was studied as a potential iron source. However, after six days 85 per cent of the iron had hydrolyzed at a pH of 8.25. These results are consistent with the experiments of Jacobson (1951) who found instability of the complex in basic aqueous solutions.

It should be noted that in all cases involving the complexing of iron, the iron and complexing agent were mixed in a 1 : 2 molar proportion. The iron concentration was 2.2 micromoles/liter.

Uptake of Iron by Asterionella japonica

In order to establish a minimal cell content of iron necessary for further growth, varying amounts of iron in the form of iron citrate were added to the culture bottles with an initial diatom inoculum of 300–400 cells/ml. The culture medium was assayed for iron colorimetrically before the addition of iron citrate. If the nutrient water showed no iron content, it was used in the growth experiments. It was normally found that inshore surface sea water filtered through a Whatman No. 42 paper was free of iron within the limit of the analytical method (0.1 micromole per liter).

The results of a typical experiment are given in Table II. No growth was observed until the initial iron concentration exceeded 0.8 micromole per liter. Both growth and final population of the cells could be controlled by the initial iron concentration. The iron per diatom, when leveling-off of the exponential growth occurred, averaged 1.0×10^{-7} micromoles per cell. We have assumed that essentially all the iron was removed by the biomass. Since the maximum cell densities were not reached until 6 or 8 days after inoculation, the greater part of the iron available to the diatoms was in particulate form.

TABLE II

Iron uptake by Asterionella japonica

Culture bottle	1	2	3	4	5	6
Initial iron in micromoles/liter	0.8	1.6	2.2	2.5	3.4	3.4
Final diatom population cells/ml.	0	14,700	21,700	18,200	41,900	41,100
Final iron content of diatoms micromoles/cell $\times 10^7$	0	1.1	1.0	1.4	0.81	0.82

Initial diatom inoculum: 300 cells/ml.
Initial phosphate: 2.62 micromoles/liter.

Humic acid has been reported as a complexing agent which makes iron more readily available to various plants and micro-organisms. Harvey (1937b) found increased growth with an artificial iron humate, but the effect was less than that of the iron citrate complex. Although the chemical formulae for the various iron complexes that are present in both natural and synthetic preparations are undetermined, it was desirable to ascertain whether iron humate might be a suitable nutrient source for *Asterionella japonica.* The synthetic humic acid and iron humate solutions were prepared according to the method of Burk, Lineweaver and Horner (1932). The iron content of the iron humate solutions was determined colorimetrically after a wet digestion with 3:1 nitric: perchloric acid mixture. The iron humate does not give centrifugable products from sea water solutions when prepared in this fashion.

The results of the humate experiments are given in Table III. Iron citrate was added to control cultures. The initial phosphate level was 2.7 micromoles per liter and the diatom inoculum was 750 cells/ml. Humate as the source of iron did not promote growth. However, the excess humic acid constituents did not deter diatom growth, as is seen in the results of bottle 5. We may therefore conclude that this form of iron humate is not available for metabolic utilization by *Asterionella japonica.*

Freshly prepared radioactive iron ascorbate was added to the culture bottles to give an initial concentration of 1.45 micromoles per liter. Three moles of ascorbate were present for each mole of iron. Exponential diatom growth was observed up to 30,000 cells per ml.; however, the cells appeared misshapen and unhealthy. No detectable iron was removed from the solution by radioactive assay of

TABLE III

Diatom growth experiments with ferric humate as the source of iron

Culture flask	1	2	3	4	5	6
Iron form	Humate	Citrate	Humate	Citrate	Citrate + Humic acid	Humate
Initial iron concentration micromoles/liter	12.5	14	12.5	14	14	6.3
Final concentration diatoms cells/ml.	0	81,000	0	62,000	70,000	0

the supernatant solution. To confirm this finding the diatoms were filtered, digested in the 3:1 nitric: perchloric acid mixture and the iron activity was determined radiometrically. The cell content of the accumulated radio-iron averaged 1.9×10^{-9} micromoles per cell. This is about 2% of the minimal iron content as determined from the uptake experiments previously described. The initial amount of particulate iron in the culture flasks was 1.8 micromoles per liter added as ferric chloride solution.

One final experiment was undertaken to confirm the observation that in the absence of complexed ferric iron and in the presence of particulate iron, diatom growth could proceed. Cells from a growing sub-culture were placed in a dialyzing sac which held 50 ml. of nutrient solution such that there was an initial diatom population of 800 cells/ml. The sac was held in place by a rubber stopper in a 250 ml. volumetric cylinder which contained 210 ml. of solution. Freshly prepared iron citrate was introduced into either the dialyzing sac or the cylinder to give an iron concentration of 2.2 micromoles per liter. In the former case the ionic complexed iron penetrated the membrane to give a concentration of 0.01 micromole per liter after 2 days, *i.e.*, 20% of the iron diffused into the outer cylinder. Here normal exponential growth occurred, whereas when the diatoms could receive only the complexed iron as in the latter case, no population increases were noted.

DISCUSSION

From the above experiments it is evident that the organic iron complexes used were not available as a growth nutrient to marine diatoms, whereas particulate and/or colloidal forms of iron are utilized in their metabolism.

Cooper (1935) found that the ratios of iron to phosphorus, in plankton samples consisting almost entirely of diatoms, were 4.2 and 4.4 in two separate determinations. From the experiments of phosphate uptake by *Asterionella japonica*, 5×10^{-8} micromoles of phosphate per cell were necessary for division. Combining this with the minimum iron content of 10×10^{-8} micromoles/cell, we calculate for our cultural populations

$$\mathrm{Fe/P} = \frac{55.85 \times 10 \times 10^{-8}}{30.98 \times 5 \times 10^{-8}} = 3.6$$

The striking agreement between these values suggests that this method of study of cultural diatom populations represents a fair simulation of ocean growth.

Cooper (1948) attributes the extreme variability in iron content in the upper layers of marine waters as due to the random distribution of particles of a large size (*i.e.*, 160 μ), as large as some sand grains. It would be of interest to know what size of iron hydroxide particle might be necessary for diatom division. We may assume a density of the particle as 1.1 and an iron content of 50 per cent. Since 1.0×10^{-7} micromoles or 55.85×10^{-13} grams are needed per cell we have, where r is the radius of the particle,

$$55.85 \times 10^{-13} = \tfrac{4}{3}\pi r^3 \times 1.1 \times \tfrac{1}{2} \quad \text{or} \quad r = 2 \times 10^{-4}\ \text{cm.} = 2\mu.$$

From the above relationships it is noted that a particle of 4 μ radius would furnish enough iron for three divisions.

The use of iron content as a parameter in a nutrient index of a productivity equation now presents itself. However, unlike necessary ionic species, the absolute concentration of iron does not reflect its availability. A direct study is needed of the number of particulate iron particles and their size distribution. One possible approach to this problem has already been initiated in this laboratory. Sea water is filtered through a cellulose membrane filter which retains particles of 0.1 μ in diameter. Following the filtration the membrane is washed with distilled water and dried. The membrane is then cleared with polyoxyethylene sorbitan monolaurate which is saturated with potassium thiocynate and is acidified with 3 drops HCl per 100 ml. The typical red iron thiocyanate color is produced about any iron particle. By the use of this technique, a study of the size distribution of the iron particles in the euphotic zone of Pacific coastal waters will be made.

SUMMARY

1. With the use of radioactive iron it was found that the marine diatom *Asterionella japonica* utilized only particulate and/or colloidal iron as a growth nutrient, whereas ionically complexed ferric ion as the citrate, ascorbate, or artificial humate was not available for uptake.

2. A minimal content of iron per cell needed for further division was established as 10×10^{-8} micromoles per liter. The ratio of the minimal iron to the minimal phosphate agrees substantially with the iron-to-phosphate ratio in natural plankton samples.

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PHARMACOLOGY OF HAEMAL VESSELS OF STICHOPUS CALIFORNICUS

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Myogenic hearts are, in general, inhibited by acetylcholine whereas neurogenic hearts are accelerated by it (Prosser, 1942). Similarly, myogenic hearts are less sensitive to ether than are neurogenic hearts (Needham, 1950). No vessels of echinoderms have previously been studied in respect to contraction mechanisms or pacemakers. The haemal vessels (lacunar system) of certain holothurians contain a fluid which is rich in protein and hemoglobin-containing corpuscles. In *Stichopus californicus,* a species often 18 inches long when extended, two large vessels serve the intestine; these are the internal vessel and the larger external vessel which is connected by many small branches to the intestine and in its lower portion also to the respiratory tree. The internal vessel appears not to be spontaneously contractile but the external vessel and its branches to the intestine contract rhythmically. The contractions are very weakly peristaltic but appear to be predominantly local, like segmentation in a vertebrate intestine. Circulation in the ramifying haemal system has not been studied in detail but the rate of flow of fluid must be very slow.

METHODS

Contractions of the external vessel were observed with a dissecting microscope and contractions indicated on a kymograph by a signal magnet. Some observations were made *in situ,* some with the vessel attached to pieces of the intestine, but most with isolated pieces of the vessel, approximately 5 cm. in length, mounted in a wax chamber through which sea water and appropriate solutions of drugs in sea water could pass at a constant rate of flow.

Rates of beat were obtained from records of a minimum of 5 contractions after equilibrium was attained with each application of a drug. Isolated preparations varied in their activity, but many continued to beat actively for several hours.

RESULTS

The frequency of beats in the external haemal vessel of Stichopus is low, ranging from 4 to 5.5 beats per minute at 18° to 20° C.

Acetylcholine strongly inhibits the beat of the haemal vessel, reversible inhibition of 16 to 36 per cent in rate occurring in three preparations at a dilution in sea water of 10^{-12}. At 10^{-14} two of four preparations showed 25 per cent inhibition. A dilution of 10^{-16} was without effect as ascertained with four preparations. At higher concentrations (10^{-10} to 10^{-6}), the beats became irregular, amplitude was markedly reduced and in the higher range the heart stopped in a relaxed state. The magnitude of the effect in the concentration range 10^{-10} to 10^{-6} varied considerably and it is not likely that the Stichopus vessel, despite its high sensitivity, would make a satisfactory assay material for acetylcholine.

Physostigmine in moderate concentrations inhibited the vessel, but a concentration of 10^{-10} in sea water had no effect alone; however, after treatment with this concentration of eserine, acetylcholine at 10^{-16}, which had previously been ineffective, caused a 25 per cent slowing.

Nicotine inhibits the Stichopus vessel. Thresholds varied, but in two preparations, significant inhibition was observed at a dilution of 10^{-11}. One preparation gave the following percentages of inhibition at the dilutions indicated: 10^{-9}, 15 per cent; 10^{-8}, 43 per cent; 10^{-7}, 57 per cent; 10^{-6}, total cessation of beat.

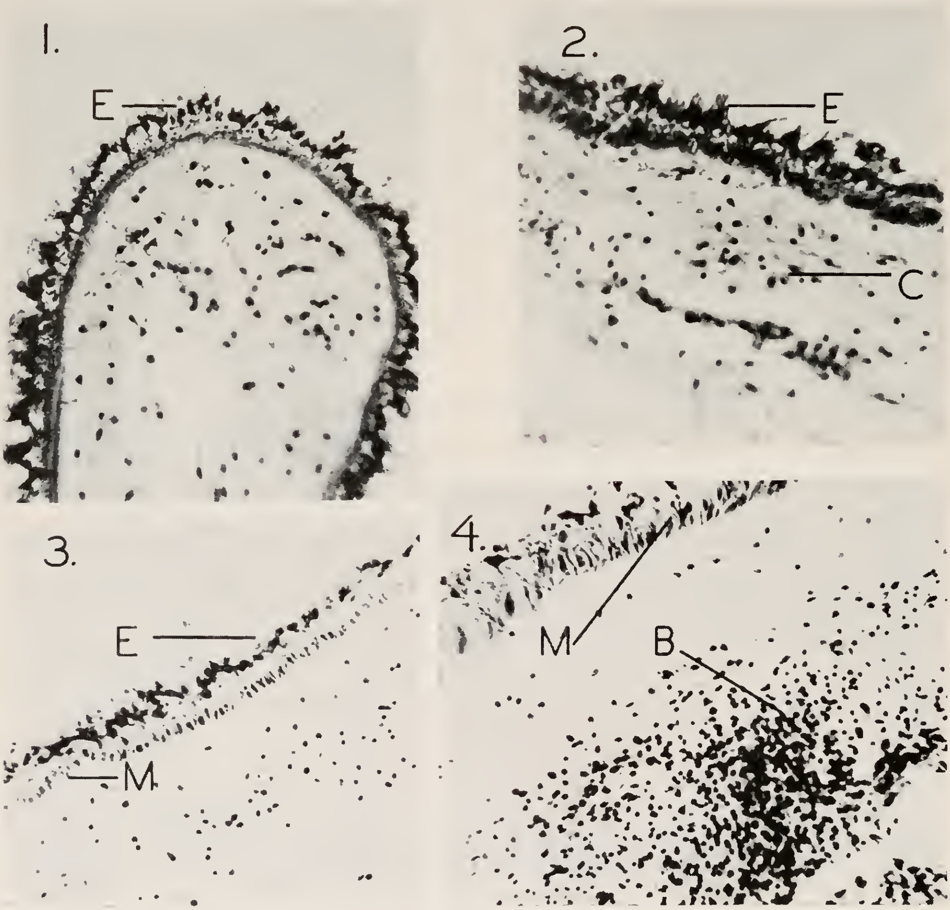

Plate I. Sections of vessels of *Stichopus californicus*. × 80. E—epithelium; M—circular muscle; C—connective tissue; B—region of formation of blood cells.

Figure 1. Cross section of small branch vessel.

Figure 2. Longitudinal section close to edge of wall of large vessel.

Figure 3. Longitudinal section through center of vessel.

Figure 4. Longitudinal section in region of hemopoiesis.

Tetramethyl ammonium bromide likewise inhibited the Stichopus vessel at concentrations of 10^{-8} and higher.

Tetraethyl ammonium bromide antagonizes the nicotinic action of acetylcholine on the heart of Venus and in mammalian sympathetic ganglia (Welsh and Taub, 1950). On the Stichopus vessel tetraethyl ammonium in concentrations which might have been effective against acetylcholine proved toxic. Dilutions of 10^{-4} stopped the vessel, 10^{-5} slowed it and 10^{-6} failed to cause recovery from acetlycholine inhibition.

The electrocardiogram of myogenic hearts usually consists of slow waves, while that of neurogenic hearts is often oscillatory (Prosser, 1950). Electrocardiograms from the haemal vessels of Stichopus were recorded with a Sanborn electrocardiograph. The vessels were mounted in mineral oil and contact made by wicks soaked in sea water. Results were extremely variable and inconstant but in several favorable preparations single slow waves of negativity were correlated with contractions. In none was oscillatory activity observed.

Histological examination of the external haemal vessel showed an outer epithelial layer, the cells of which have small protruding processes (Figs. 1, 3). These projections give a villous appearance to the epithelium and greatly increase the surface exposed to the body cavity. Beneath the epithelium is a single layer of circular muscle fibers but no longitudinal muscle fibers (Figs. 1, 2, 3); this observation explains the segmental nature of the contractions. Most of the vessel is composed of large, loosely-packed connective tissue cells which contain regions of blood-cell manufacture (Fig. 4). The lumen is lined with a thin unicellular layer of endothelium. It cannot be said positively that nerve cells are absent from the vessel, but certainly no aggregations of them occur.

CONCLUSIONS

The external haemal vessel of the holothurian, *Stichopus californicus,* beats spontaneously. It consists of villous epithelium, circular muscle, and connective tissue with regions of hemopoiesis. The rhythmic contractions are inhibited by acetlycholine with a threshold concentration of the order of 10^{-14}. Physostigmine potentiates the response to acetylcholine while both nicotine and tetramethyl ammonium chloride inhibit the beat of the vessel. The action potential appears to be a simple wave of negativity. By analogy with the pharmacology of other hearts it is tentatively concluded that the Stichopus haemal vessel is myogenic.

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CYTOLOGICAL STUDIES ON MUCUS FORMATION AND SECRETION IN BUSYCON [1]

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The formation and secretion of mucus, sometimes of great importance to physiologists and ecologists, are poorly understood. A few studies have been made of mucous glands and of their activity in various organisms. Thus, Re (1951) has studied secretion of mucus in the amphibian oviduct, and Patten (1950) has described some aspects of histogenesis in mucous epithelia of a urodele and two rodents. Vaubel (1933) observed the behavior of mammalian synovial cells in tissue culture. Studies on invertebrates have included observations on some cytological aspects of the secretion *in vivo* by an oligochaete (Millott, 1948). The mucus-producing ability of mollusks is well known; the pallial mucous cells of a pelecypod, *Anodonta*, have recently been studied by Defretin and Riff (1948). The prosobranch (gastropod) hypobranchial gland contains masses of large mucus-producing cells, and for this reason deserves special attention (Ronkin and Ronkin, 1951). Thus Tarao (1935) described the hypobranchial gland of the abalone, *Haliotis japonica*, and attempted to analyze the time-sequence of the secretion using Hirsch's (1931) methods for the classification and analysis of secretory activity. An earlier morphological description of the gastropod hypobranchial gland (Dakin, 1912) includes some reference to previous work. The present study is largely cytochemical in nature and is directed mainly toward an understanding of the chemical processes leading to formation of mucus in the hypobranchial gland of *Busycon*. Since this gland has not been adequately described, it is necessary to include a brief morphological survey; this adds to our meager knowledge of molluscan histology.

MATERIALS AND METHODS

Obtaining the excised gland. *Busycon canaliculatum* (Linnaeus), a large marine snail, is easy to handle and was readily available during most of the year by dredging near Woods Hole, Mass. To obtain the fresh gland the animal was held on a board, with the columella vertical and apex pointed downward. The outer whorls of shell were cracked with a hammer; then a *dulled* axe blade was inserted into the external groove separating adjacent whorls, and twisted. After one or two whorls of shell were removed in this manner the animal was grasped at the operculum, its columellar muscle worked loose with the fingers, and the intact snail then "unscrewed" from the remaining shell. One could now make out the pallial organs

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[2] With the technical assistance of Elizabeth P. Ronkin.

(rectum, hypobranchial gland, ctenidium and osphradium, from the midline to the animal's left), through the translucent roof of the pallial cavity. The portion of the mantle bearing the hypobranchial gland was excised for study.

Preparation of material for microscopic study. The excised gland was easily maintained alive in sea water at room temperature (20 to 25° C.) for five days, during which time ciliary activity and secretion of mucus were evident. Attempts to obtain separate, living mucous cells and to observe them with the compound microscope failed, probably because of the fragility of these cells. For the same reason, attempts to obtain fresh-frozen sections with the freezing microtome also failed. However, it was possible to obtain frozen sections (at 25 μ) of glandular epithelium which had previously been fixed in 10% neutral formalin for 8 to 24 hours. Some attempts to stain the living cells with dilute solutions of neutral red, Janus green B, and toluidine blue O were also made, but the dye failed in each case to penetrate the mucous layer surrounding the cells. For most histological and cytological studies the gland was placed in a fixative solution immediately after excision.

Gilson's mercuric-nitric solution gave good fixation most often when compared with other fixatives (acetone at 5° C., alcohol-formalin, Formol-Zenker, *i*-pentane at − 170° C., lead acetate-formalin, 5% mercuric chloride, osmic-sublimate,[3] propylene glycol at − 20° C., Regaud's, Susa's, and toluene at − 20° C.). Ethanol was used for dehydration, followed by clearing in toluene and infiltration and imbedding in paraffin. Sections were cut at 7 to 10 μ.

For general studies of histological and cytological features, sections were stained with toluidine blue O (0.1% aqueous) followed by potassium ferrocyanide (1% aqueous), Weigert's hematoxylin, and metanil yellow (0.25% in 0.25% acetic acid), with the usual intermediate rinses and subsequent dehydration, clearing, and mounting. Mayer's mucicarmine was occasionally used as a substitute for the toluidine blue O-potassium ferrocyanide combination, and was satisfactory for many purposes. Mallory's triple stain was used for histological features.

In addition to these, and to certain special methods described in the literature, the following methods are referred to in this paper:

a. Alcian blue 8GS.[4] This stain, whose use for mucus has recently been suggested by Steedman (1950), was employed in 1% aqueous solution; metanil yellow was used as a counterstain.

b. Amylase digestion. Hydrated sections were incubated in human saliva at 37.5° C. for one hour, then for an additional half-hour in distilled water to remove the salivary mucus.

c. Bauer-Feulgen stain. This procedure for demonstrating glycogen and mucus follows the schedule outlined by Glick (1949), using 4% chromic acid.

d. Hyaluronidase digestion. Hydrated sections were incubated in 0.01% bovine testicular hyaluronidase[5] at 37.5° C. for the desired time. They were then rinsed several times in distilled water before further treatment.

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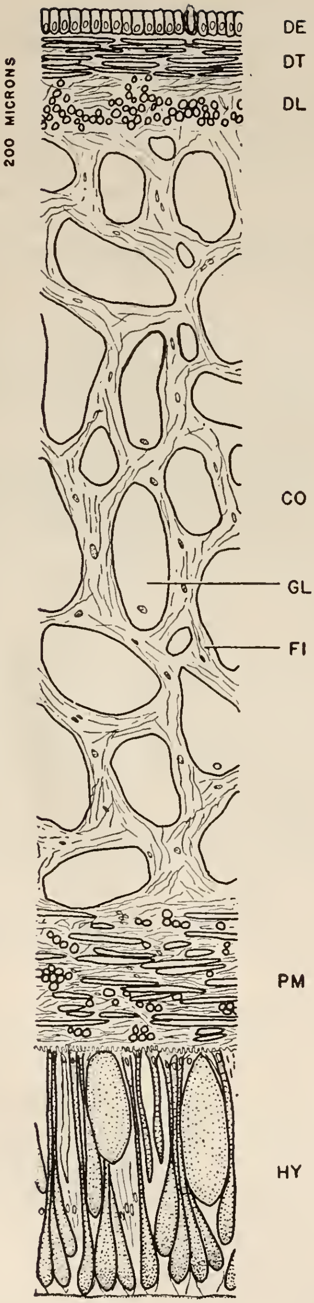
[3] Ludford-Mann-Kopsch method (Bensley and Bensley, 1938).

[4] A new dye derived from monastral fast blue B (Haddock, 1948). Samples were kindly furnished by Dr. H. A. Lubs of E. I. du Pont de Nemours and Co., Inc., and by Dr. H. F. Steedman of the University of Glasgow. It has recently become commercially available as a biological stain in the United States.

[5] Worthington Biochemical Sales Co., Freehold, N. J.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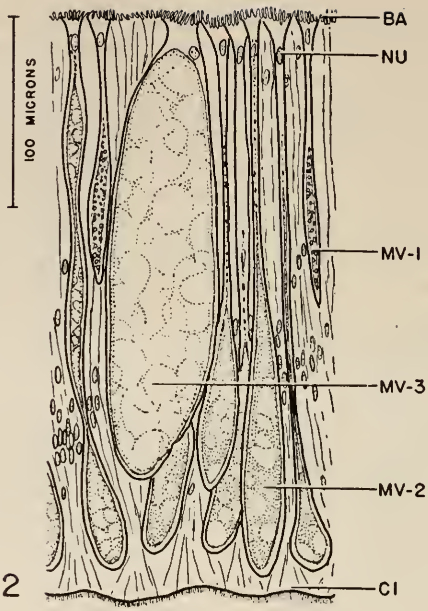
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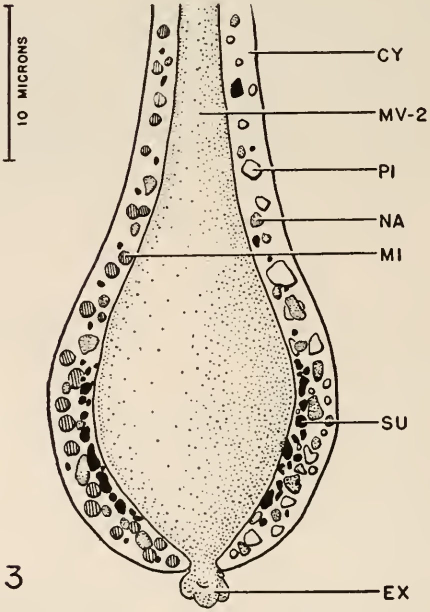
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3

FIGURE 1. Semi-diagrammatic transverse section through a portion of the mantle of *Busycon* bearing the hypobranchial gland. CO, connective-tissue layer; DE, dorsal epithelium; DL, dorsal longitudinal muscular layer; DT, dorsal transverse muscular layer; FI, fibrous tissue; GL, glycogen cell; HY, hypobranchial gland; PM, pallial muscular layer.

e. Nadi test. This procedure for demonstrating cytochrome oxidase, using α-naphthol and p-amino dimethylaniline, is described by Glick (1949). Tissues were fixed in 10% formalin and sectioned with the freezing microtome, stained, and mounted in Apáthy's gum-syrup (Lillie, 1948).

f. Prussian-blue test for intracellular iron. Hydrated sections were immersed first in ammonium sulfide, then in potassium ferrocyanide solution (0.75% in 0.25% HCl), and finally counterstained in eosin Y, with intermediate rinses in distilled water.

When available, Commission-certified stains were used. Unless otherwise stated, stained sections were dehydrated in a series of ethanols, cleared in xylene and mounted in Clarite. When enzymatic digestion was used, comparisons were made with undigested sections from the same block.

OBSERVATIONS

General structure of the hypobranchial gland (Fig. 1). The glandular and ciliated epithelium which faces the pallial cavity consists of a single layer of long, slender cells, arranged normal to the free epithelial surface, and is bounded dorsally by a basement membrane. Above the basement membrane is a relatively thin sheet of fibrous and muscular tissue, with fibers arranged parallel to the free surface. The thickest layer of the mantle comes next and consists of a loose network of fibrous connective tissue. Most of the spaces bounded by the fibers are filled by cells which contain glycogen in large amounts. Next dorsally is a muscular and connective-tissue layer. Finally there is a very thin layer of glandular epithelial cells which bounds the dorsal (outer) surface of the mantle.

Examination of the inner epithelial layer (*i.e.*, the hypobranchial gland itself; Fig. 2) revealed that it is composed of ciliated cells, several types of mucous cells, and some rather interesting cellular fragments. The ciliated cells are conical. The bases of the cones pave the surface of the gland and their apices are prolonged as slender stalks whose extremities probably attach to the basement membrane. The nucleus of each ciliated cell is to be found about half the distance from the surface to the basement membrane.

Types of mucous cells. The following working classification of mucous cells is proposed:

Mucous cells of type 1 frequently extend only part way from the basement layer to the free surface of the gland. The mucous vacuole is slender and usually spindle- or club-shaped; its contents appear gray and foamy in unstained material, dark red with Mayer's mucicarmine, and frequently blue (*i.e.*, without metachromasy) with toluidine blue O.

FIGURE 2. Cell types in the hypobranchial gland. BA, basement membrane; CI, ciliated cells; MV–1, 2, 3, mucous vacuoles of cells of types 1, 2, and 3, respectively; NU, nucleus of a type-2 cell. Note the masses of nuclei about two-thirds the distance from the basement membrane to the ciliated surface.

FIGURE 3. Semi-diagrammatic drawing of the distal end of a type-2 cell. CY, cytoplasm; EX, extruded mucus; MI, mitochondrion (mitochondria are cross-hatched and are omitted from the right half of the drawing); MV–2, mucous vacuole; NA, nadi-positive granule (stippled); PI, pigment granule (omitted from the left half of the drawing); SU, sudanophilic granule (solid black). Golgi material is not represented.

Mucous cells of type 2 generally extend the full depth, or nearly the full depth, of the glandular layer. The large mucous vacuole is club-shaped, and usually can be followed from its distal enlargement almost to the base of the cell; although one or two broad regions may be seen, usually the vacuole tapers rapidly and terminates in isolated granules close to the nucleus. In some cells the nucleus is at some distance from the basement membrane; in such cells all the mucus is distal to the nucleus, and the cell is connected to the basement layer by a slender stalk. The mucus stains metachromatically (reddish violet) with toluidine blue O and bright red with mucicarmine; it appears gray and foamy in unstained material. One may suggest that cells of type 1 include the youngest in a series of mucous cells of different ages, and that type-2 cells are older; evidence in support of this relationship will be offered elsewhere in this paper.

TABLE I

Staining and other reactions of mucous-vacuolar contents in the various kinds of glandular cells described

Stain and condition	Type 1	Type 2	Type 3
a. Mayer's mucicarmine	Reddish violet	Red	Like type 2
b. Alcian blue 8GS	Blue-green, but parts of some vacuoles unstained	Blue-green	Like type 2
c. Toluidine blue O	Some blue and nongranular; some blue with fine or coarse metachromatic granules; the latter having decreasing amounts of blue-staining interstitial material; some with metachromatic, foamy contents with optically empty interstices	Reddish violet (metachromatic)	Like type 2
d. Toluidine blue O, preceded by 24 hours hyaluronidase digestion	Metachromasy in granules, but paler; blue color faded or absent	Pale metachromasy	Like type 2
e. Bauer-Feulgen	Red granules, of uniform size within each cell	Red and finely granular	Clumps of fine, pink granules
f. Bauer-Feulgen, preceded by 24 hours hyaluronidase digestion	No visible change on exposure to enzyme	Pale pink	Optically empty

Mucous cells of type 3 have very large, ellipsoidal or irregular vacuoles whose contents, when present, appear to have the same staining properties as those of type 2, but seem to be less dense or more open in texture. They are found close to the basement layer but may extend most of the distance to the surface. Whether cells of type 3 are part of the same series as those represented by types 1 and 2 cannot be stated for lack of evidence. However, the similarity between types 2 and 3 with respect to staining reactions and susceptibility to hyaluronidase (Table I) suggests chemical similarities in the mucus.

Usually the mucous cells are normal to the free surface, but some portions of the gland (especially those at its left, or ctenidial, edge) are thrown into folds which bear a superficial resemblance to the gastric crypts in vertebrates. In such locations the mucous cells usually appear to be of type 2, but they are much shorter than elsewhere in the gland and seem to pour their secretions into the crypt rather than directly into the pallial cavity.

Masses of nuclei, paler and more elongated than those found at the bases of mucous cells, were frequently seen occupying clusters of cytoplasmic strands, at positions about halfway from the basement membrane to the free surface. Many nuclei were also encountered in the secreted mucus lying on the surface of the gland. Some of these nuclei belong to ciliated cells, but none could be traced to mucous cells. It is possible that the remaining nuclei and cytoplasmic strands are remnants of former mucous cells whose distal portions have become emptied or lost.

The contents of the mucous vacuoles. In formalin-fixed material, frozen-sectioned and unstained, the contents of the mucous vacuole are gray, refringent, and appear foamy in texture. In toluidine blue-, alcian blue-, or mucicarmine-stained cells the appearance is slightly distorted, probably owing to the preceding dehydration, and the contents tend to look fibrous. It is the fibrous phase, corresponding to the continuous or suspending phase of the "foam," which becomes stained with uniform intensity throughout the vacuole. It is thus unlikely that the suspended spheres in the mucous vacuole, which remain unstained, are of mucous nature. The mucus in the three types of mucous cells stains differently with the various staining methods used, as indicated in Table I.

Cytoplasmic inclusions other than mucus (Fig. 3). The fresh gland appears yellowish brown to the unaided eye. Microscopic examination of portions of the gland fixed in 10% formalin and sectioned with the freezing microtome revealed that the scanty cytoplasm of the stained or unstained mucous cells of all types contains many canary-yellow granules. The yellow color which they confer upon the cytoplasm is visible under low power. These granules vary widely in size, the largest being about 5 μ in diameter and the smallest at the limit of microscopic resolution in white light. The larger granules appeared to be concentrated about the distal, more bulbous portion of each type-2 cell. The following description refers to type-2 cells, which are those most commonly found.

Mitochondria were demonstrable with Regaud's hematoxylin. Spherical basophilic granules, 0.4 to 2.1 μ in diameter, were concentrated and more easily distinguishable in the distal half of the mucous cell than in the proximal, or basal, half. They were not as easily demonstrated with Altmann's aniline acid fuchsin.

The possible presence of Golgi material was shown by osmiophilia in the mucous cells of deeper portions of the "crypts" at the left edge of the hypobranchial gland. The densely packed, intensely brownish-black granules occupied the distal portion of the cytoplasm of each cell, outside the mucous vacuole. Since these sections had been bleached with potassium permanganate subsequent to osmic-acid treatment, the distribution of osmiophilic granules may indicate the location of Golgi material (Lillie, 1948).

Formalin-fixed, frozen-sectioned glandular material stained with Sudan black B (Wislocki and Dempsey, 1948) revealed blackish-green granules distributed throughout the cytoplasm, but concentrated especially in a broad belt encircling the distal portion, but not the tip of the mucous vacuole. Most of the sudanophilic granules were 0.4 to 1.3 μ in diameter, with a few as large as 2.5 μ. Several belts of granules measured were 4 to 12 μ wide, compared with mucous-vacuolar diameters of about 17 μ in this portion of the cell. Sudanophilia is presumptive evidence of lipid nature.

Similarly fixed and sectioned material was treated with the "nadi" reagent for the determination of cytochrome oxidase. Treated sections showed intense, blue granules, usually varying in diameter from 1.7 μ downward, throughout the cytoplasm. Near the expanded distal portion of the cell, granules up to 3.5 μ in diameter were found.

Iron was not present in any of the mucous cells in amounts large enough to be detected by the Prussian-blue test. Prussian-blue granules were present, however, in the ciliated epithelium of the adjacent ctenidium, portions of which were sometimes included on the same slides with hypobranchial-gland material.

Attempts to demonstrate glycogen in the mucous cells with the Bauer-Feulgen test were unsuccessful. The presence of Bauer-Feulgen-positive material in the underlying connective tissue, and its absence in control sections which had been subjected to digestion with salivary amylase, suggested that glycogen was absent from the mucous cells.

Observations of secretion. A few cells were observed in which the secretion of mucus had been stopped by histological fixation. The freshly extruded mucus usually appeared as a small wisp protruding from the center of the distal tip of the cell, but occasionally as a small rounded cap or button covering the tip of the cell. There was no evidence of a large amount of mucus being extruded from a cell in a short time, suggesting that the production of mucus is a slow and continuous type of activity, probably varying in rate from time to time, and with secretion occurring only occasionally. Millott (1948) has seen sphincter-like structures surrounding the mouths of mucous cells in *Lumbricus;* no analogs of these structures could be seen in material studied here. It is possible that the ciliated cells, by the lateral exertion of pressure, may aid in mucus secretion in *Busycon* in the way Millott has suggested for the oligochaete, but no evidence for this mechanism was observed.

Discussion

If one defines a mucous cell for the purposes of this study as one whose secretion-vacuolar contents stain metachromatically with toluidine blue O, then it is probable that all kinds of secreting cells observed were mucous in nature. In *Haliotis,* however, Tarao (1935) found several kinds of non-mucous, secreting cells which he was able to classify according to the types of secretion granules. The non-mucous cells were intermixed with mucous, ciliated, and sustentacular cells in the hypobranchial gland. In *Busycon* all the observed glandular cells appear to be associated with mucus production, and no sustentacular cells could be distinguished, though these may have been represented by some of the massed nuclei referred to above.

The probable involvement of the nucleus in mucus formation, suggested by Tarao, is supported by the location of the nucleus near the slender base of the mucous vacuole in *Busycon.* Painter (1945) has suggested that formation of secretory materials is related to nuclear metabolism in Drosophila and in the honeybee. Wilson (1928) has referred to the earlier literature on nuclear function in relation to secretory activity in protozoa.

The genetic relationships of the different kinds of glandular cells in *Busycon* do not seem as clear as those described in *Haliotis* by Tarao. The observations reported here suggest that mucous cells of types 1 and 2 are earlier and later stages in the maturation of cells of a single type. The position of cells of type 3 is uncertain; they may constitute a separate category, or they may represent an older stage than type 2, in the same series. The evidence for the relation between cells of types 1 and 2 lies in (1) the generally greater length of type-2 cells, compared with type 1; (2) the failure to observe extrusion of secretion from type-1 cells; (3) the heterogeneity of vacuolar contents of type 1, with respect to its staining reactions with alcian blue and toluidine blue, suggesting a transitional state.

The last observation confirms those of Vaubel (1933), who found that young synovial cells in tissue culture contained granules orthochromatic to toluidine blue, whereas older cells contained metachromatic granules. In *Busycon* the acquisition of metachromasy by the mucous-vacuolar contents, which is apparently in progress in type-1 cells, reflects a chemical change in the mucous precursor. The property of metachromasy is correlated in general with the binding of sulfate in polysaccharides (Lison, 1933; see also Michaelis and Granick, 1945, and Masamune *et al.,* 1947), and it seems likely that sulfate binding may be an important step in the formation of mucus in progress in cells of type 1.

The reported presence of glycogen in the mucous cells of *Anodonta* (Defretin and Riff, 1948) indicates a difference between *Busycon* and this pelecypod material. The failure to detect glycogen in the hypobranchial mucous cells and its presence in the overlying tissues suggest that the mucous cells may obtain their carbohydrates by diffusion from the neighboring cells. The presence of galactose in other molluscan tissues (Bell and Baldwin, 1941; Masamune *et al.*, 1947) may mean that galactogen is of general importance in this group of animals, in addition to glycogen; galactokinase and galactose-isomerase systems might well be sought.

Finally, the peculiar distribution of lipid material in relation to the mucous vacuole in type-2 cells suggests that lipid metabolism may be involved in the formation or secretion of mucus, or both. Although the granules which appeared in these cytochemical tests may well be crystalline precipitates and may not exist in the living cell, their locations suggest the distribution of constituents present in life. The distribution of mitochondria and that suggested for cytochrome oxidase may indicate the loci of biochemical systems involving aerobic syntheses. Studies on lipid transformations and respiratory metabolism in these cells and further studies of the spatial distribution of the participating substances may clarify the mechanisms of the formation and secretion of mucus.

SUMMARY

1. The hypobranchial (mucous) gland of *Busycon canaliculatum* (L.) is located in the pallial cavity. It contains ciliated cells and three types of mucous cells.

2. Mucous cells of type 1 are thought to be precursors of type 2 because of the relative sizes and positions of the two types of cells, and because mucous vacuoles of type-1 cells appear to have varying degrees of resemblance to those of type-2 cells. Evidence concerning the relationships of type-3 cells is lacking.

3. The commonest type of mucous cell (type 2) is long and slender with a bulbous tip, one or two swellings along its length, and a basal nucleus. Most of the cell is occupied by a mucous vacuole, whose contents stain metachromatically (reddish violet) with toluidine blue O, red with mucicarmine, blue-green with alcian blue 8GS, and are partly digested by hyaluronidase. The surrounding cytoplasm con-

tains yellow pigment granules, mitochondria, Golgi apparatus, and nadi-positive granules. The distribution of sudanophilic material is peculiar in that a belt of lipid granules surrounds the sub-distal portion of the bulbous tip of the vacuole. No glycogen could be demonstrated in these cells.

4. It is possible that the activities of mucous cells require diffusion of carbohydrate materials from adjacent cells. The results of the cytochemical tests suggest that aerobic utilization of carbohydrates (possibly galactose) or lipid materials, and the binding of sulfate groups may be of importance in the formation of mucus from its precursors.

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NEUROSECRETION. XI. THE EFFECTS OF NERVE SECTION ON THE INTERCEREBRALIS–CARDIACUM–ALLATUM SYSTEM OF THE INSECT LEUCOPHAEA MADERAE[1]

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In the course of a histological study of the intercerebralis-cardiacum-allatum system in specimens of the insect *Leucophaea maderae* in which the nervi corporis cardiaci had been sectioned (B. Scharrer, 1946a), it became evident that this system offers a favorable material for the investigation of a question of general importance, namely that of the transport of neurosecretory material along nerve fibers.

The intercerebralis-cardiacum-allatum system of insects is in many ways analogous to the hypothalamo-hypophyseal system of vertebrates (Hanström, 1941; M. Thomsen, 1943; Scharrer and Scharrer, 1944; Cazal, 1948). In both cases histological observations suggest that the stainable material produced by the neurosecretory cells of the pars intercerebralis and the hypothalamus, respectively, passes along the axons of these cells into organs in which it is stored. In the insects these axons form the nervi corporis cardiaci (nervi corporis cardiaci I, Hanström) and reach not only the corpora cardiaca but in part also the corpora allata. The corpora cardiaca act in the capacity of storage organs for neurosecretory material, in at least a number of species (B. Scharrer, 1951; 1952c); in the vertebrates the neurohypophysis fulfills a corresponding function (Bargmann and Scharrer, 1951).

If the neurosecretory substance actually "migrates" along the axons of the cells from which it originates, it might accumulate when its flow is blocked, as for instance by section of the nerve fibers. Hild (1951), working with frogs, and Stutinsky (1951), using frogs and rats, were indeed able to demonstrate the accumulation of neurosecretory material in the pituitary stalk proximal to the site of section. Scharrer and Wittenstein (1952) reported corresponding observations in dogs in which the pituitary stalk had been severed. However, when compared with insects, vertebrates do not offer equally favorable conditions for the study of this problem. In the operation on vertebrates not only nerve fibers, but also blood vessels must, by necessity, be severed. It is difficult to assess the part played by the interruption of the vascular channels in causing histological changes in addition to those following the severance of the nerve fibers. In the intercerebralis-cardiacum-allatum system of insects the effect of the interruption of the fiber tracts can be studied without reference to changes that might be caused by interference with the blood supply. A further advantage is that the intercerebralis-cardiacum-allatum system is paired in many insects, including Leucophaea. Consequently, the neurosecretory pathway can be sectioned on one side without damage to the other side which serves as control in the same animal. The effects of the interruption of this neurosecretory

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pathway were studied, therefore, in Leucophaea with regard to (a) the transport of the stainable material produced by neurosecretory cells along the axons of these cells, (b) the storage function of the corpora cardiaca, and (c) the reaction of the corpora allata.

MATERIAL AND METHODS

Nerve sections were performed in 124 nymphs and in 115 adults of Leucophaea of both sexes. The nervi corporis cardiaci which enter the corpora cardiaca soon after emerging from the postero-ventral surface of the brain are not easily accessible. The most suitable approach was found to be through a window cut anteriorly in the head capsule; the brain had to be somewhat tilted so that the nerves could be severed. As a rule the operation was performed in nymphs at the beginning of an intermolt period and in adults shortly after emergence. The operated specimens were allowed to survive from 5 days to 10 months. The heads were fixed in Zenker-formol or Bouin, and serial sections of 5 micra were stained with Gomori's (1941) chrome alum-hematoxylin-phloxin method or with Foot's (1933) modification of Masson's trichrome stain. While the Gomori technique is not more specific for the identification of neurosecretory material than the Masson method, it affords a more precise characterization of this substance in both vertebrates and invertebrates than the Masson and other previously used methods and is, therefore, preferable. The sections on which the present study is based include cases with unilateral and with bilateral interruption of the nervi corporis cardiaci.

Since the amount of neurosecretory material normally present in the intercerebralis-cardiacum complex varies to some degree with age, physiological conditions, etc., the cases with unilateral nerve section are particularly instructive for the determination of the histological changes resulting from the interruption of the neurosecretory pathway.

OBSERVATIONS

The effects of the severance of the nervi corporis cardiaci on the histological appearance of the intercerebralis-cardiacum-allatum system of Leucophaea are twofold. One result of this operation consists in marked effects on the distribution of the neurosecretory material, the other concerns a structural change in the corpora allata. Male and female animals respond to the operation in the same manner.

The topography of the organs in question as well as the levels at which sections for photomicrographs (Figs. 2–9) were selected are indicated diagrammatically in Figure 1. The paired nervi corporis cardiaci which originate from the neurosecretory cells of the pars intercerebralis cross before leaving the brain. Their axons enter the elongated corpora cardiaca where they can be traced on each side for a considerable distance as a well defined nerve bundle. In all probability some of the fibers reach the corpora allata, but since in Leucophaea the corpora allata are directly attached to the posterior end of the corpora cardiaca, there is no distinct nervus corporis allati.

As has been pointed out previously (Scharrer and Scharrer, 1944) the distinctive feature of the nervous pathway connecting the pars intercerebralis of the brain with the corpora cardiaca-allata is the presence of varying amounts of neurosecretory material throughout its course. Within the corpus cardiacum this material, which stains deep blue with the Gomori technique and bright red with the Masson stain,

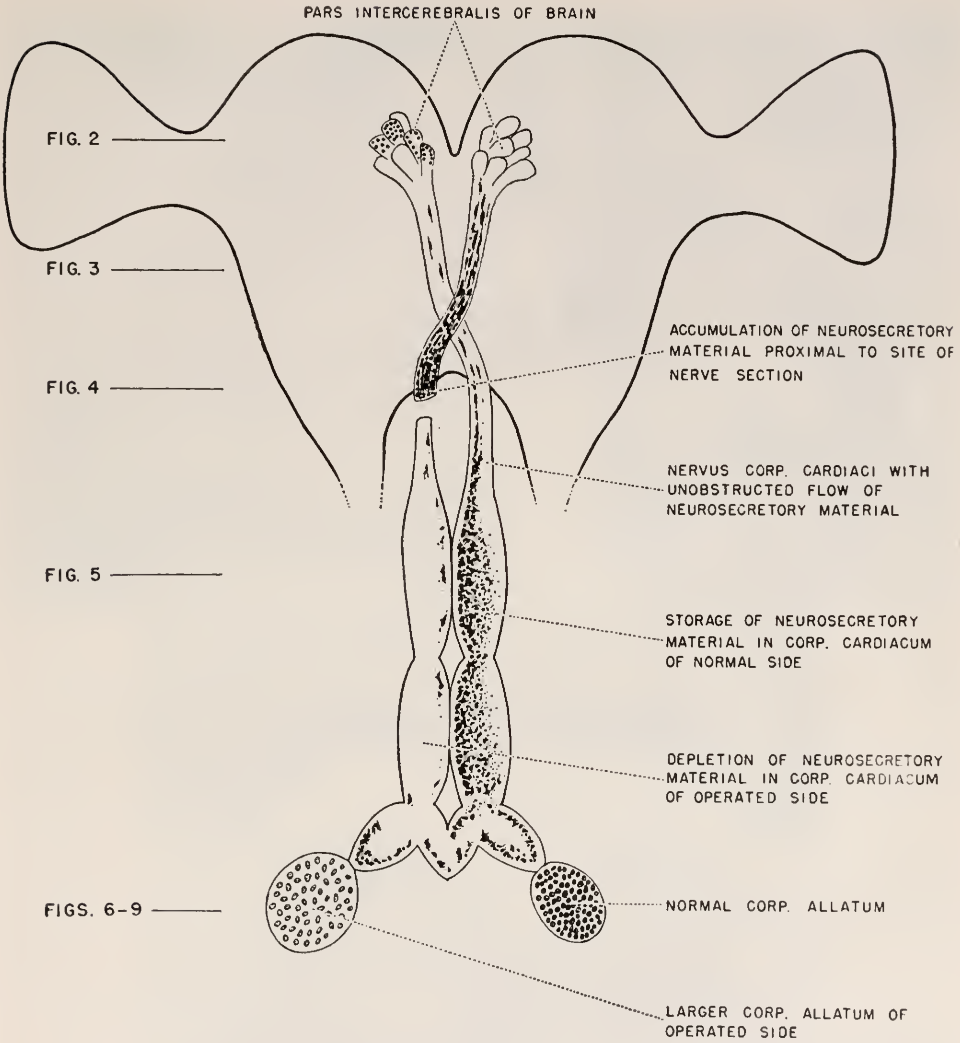
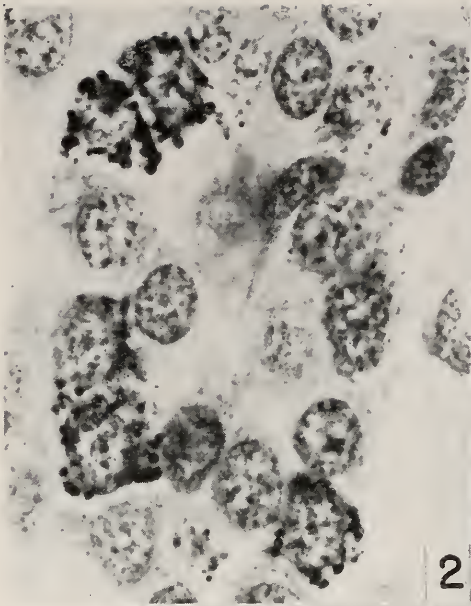

PARS INTERCEREBRALIS OF BRAIN
FIG. 2
FIG. 3
FIG. 4
FIG. 5
FIGS. 6–9
ACCUMULATION OF NEUROSECRETORY MATERIAL PROXIMAL TO SITE OF NERVE SECTION
NERVUS CORP. CARDIACI WITH UNOBSTRUCTED FLOW OF NEUROSECRETORY MATERIAL
STORAGE OF NEUROSECRETORY MATERIAL IN CORP. CARDIACUM OF NORMAL SIDE
DEPLETION OF NEUROSECRETORY MATERIAL IN CORP. CARDIACUM OF OPERATED SIDE
NORMAL CORP. ALLATUM
LARGER CORP. ALLATUM OF OPERATED SIDE

FIGURE 1. Diagram of the dorsal aspect of the intercerebralis-cardiacum-allatum system of *Leucophaea maderae.* On the left side the nervus corporis cardiaci is severed with the result that neurosecretory material is increased proximal to and depleted distal to the site of nerve section. On the operated side the corpus cardiacum is decreased, the corpus allatum increased in size. Levels of the photomicrographs shown in Figures 2–9 are indicated; the sections illustrated in these figures were made from paraffin-embedded material, cut at 5 micra, and stained with the chrome alum-hematoxylin-phloxin method of Gomori.

follows in its distribution the course of the fiber bundles of the nervi corporis cardiaci. This topographic relationship is particularly evident in the anterior portion of the corpora cardiaca. Masses of neurosecretory material are usually confined to this nerve bundle for some distance and, farther distally, "fan out" into the cardiacum tissue. Neurosecretory material is, as a rule, more abundant in the marginal zones,



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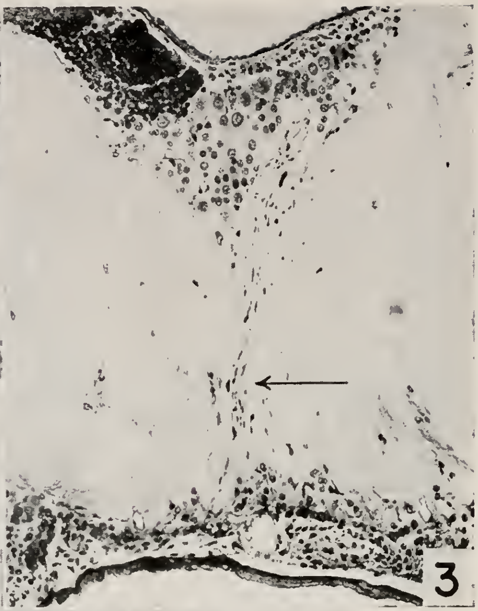

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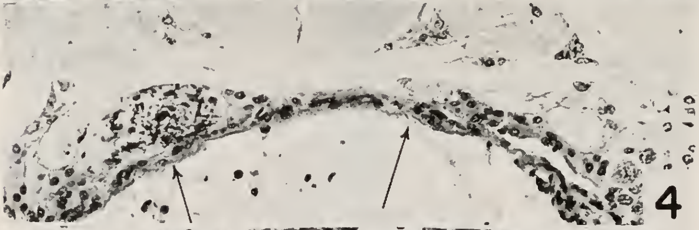

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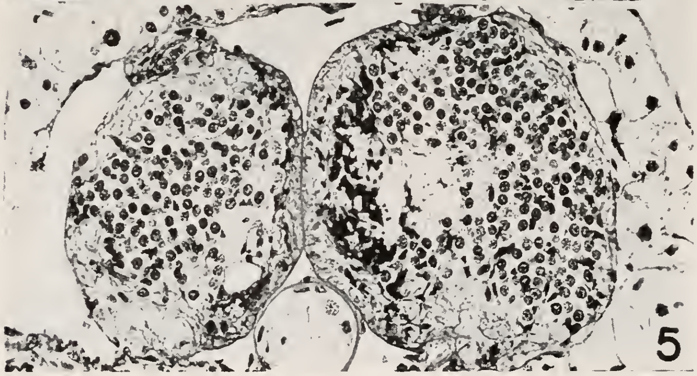

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FIGURE 2. Neurosecretory cells of the pars intercerebralis of Leucophaea on the unoperated side with conspicuous cytoplasmic inclusions. The operated side of this case shows very little neurosecretion. Adult female, fixed in Bouin 5½ months after operation. Photomicrograph, × 1000.

FIGURE 3. Brain of adult female fixed in Bouin five months after operation. Level of decussation of nervi corporis cardiaci. At the arrow the tract of the operated side, which contains an increased amount of dark staining neurosecretory material, crosses the almost empty tract of the intact side (compare with Figure 1). Photomicrograph, × 100.

particularly those facing the lumen of the dorsal vessel which is surrounded by the corpora cardiaca. In the same sections the corpora allata are free of neurosecretory substance.

1. *The effect of the severance of the nervi corporis cardiaci on the distribution of neurosecretory material*

If the neurosecretory pathway is interrupted in the manner described, the result is an accumulation of neurosecretory material proximal to the site of operation and a marked depletion of this substance in the distal portions of this system (B. Scharrer, 1952a). These histological changes suggest that the normally occurring flow of neurosecretory material along the axons of the cells in which it originates must have been blocked by the nerve section.

The accumulation of neurosecretory material at the proximal end of the cut fiber tract is particularly noticed in cases where the interval between the operation and the fixation of the tissues is short, *i.e.*, several days. Figure 4 shows a cross section through the nervi corporis cardiaci near the ventral surface of the brain a short distance from their exit. On the side where the operation was performed the nerve contains considerable masses of neurosecretory material, while the nerve on the unoperated side contains only a small amount. In cases where the interval between the operation and the fixation of the organs is several months, the accumulated neurosecretory substance usually extends to a more proximal point with the result that the severed tract, throughout its course within the brain, shows an increased amount of inclusions as compared with the control side (Fig. 3). This phenomenon of accumulation of the substance is also observed in cases of bilateral nerve section which serve to corroborate the results of unilateral operations. In specimens which had been allowed to survive for the longest period of time in this series of experiments (up to 10 months) the accumulation effect may be absent. It is to be assumed that the neurosecretory cells whose axons have been cut eventually cease to produce, or produce a smaller amount of the material which is normally transported to the corpora cardiaca. This assumption is supported by the observation that in some of the long interval cases studied the cells of the pars intercerebralis contain more neurosecretory material on the normal than on the operated side (Fig. 2).

While the section of the nervi corporis cardiaci results in an increase of neurosecretory material in the proximal part of the system, the portions distal to the level of transection show a reduction in the amount of this substance. Figure 5 demonstrates the difference between normal and operated side in that the corpus cardiacum whose nerve has been severed contains practically no material stainable with the

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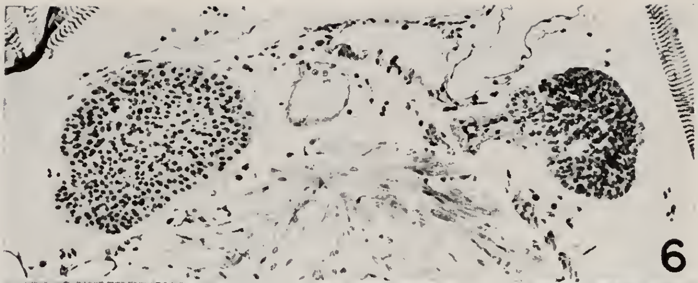
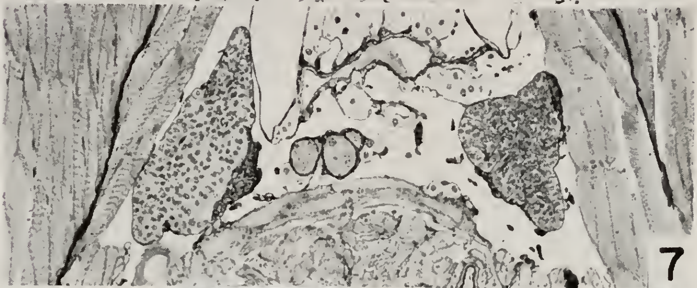
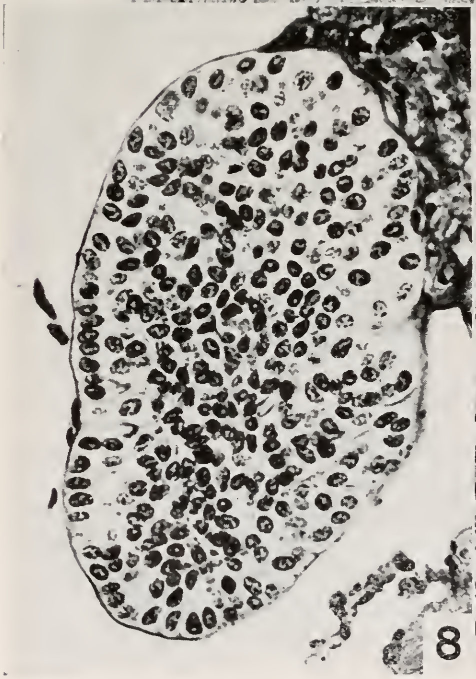
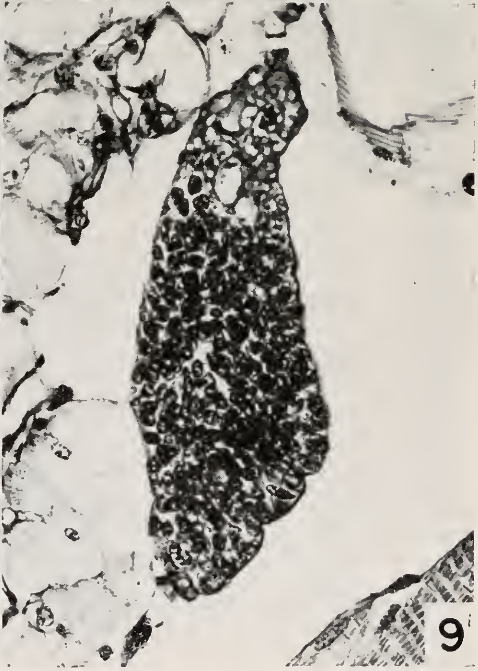
FIGURE 4. Cross section of nervi corporis cardiaci (arrows) in postero-ventral part of brain near site of their emergence. The severed tract on the left side, shown proximal to the level of operation, contains considerably more dark staining material than the intact tract on the right side. Female fixed in Zenker-formol five days after nerve severance. Photomicrograph, × 160.

FIGURE 5. Cross section of corpora cardiaca of female with unilateral severance of nervus corporis cardiaci, operated five months before fixation in Bouin. The corpus cardiacum on the operated (left) side is smaller and contains very little neurosecretory material in contrast to the normal control gland on the right side which shows dark masses of this substance in the medial zone. Photomicrograph, × 210.









FIGURES 6–9. Corpora allata of Leucophaea with unilateral severance of the nervus corporis cardiaci. The organs on the operated side at left are "swollen" with more cytoplasm and looser arrangement of nuclei than those on the normal right side. Figure 6: Female fixed in Zenker-formol five days after nerve severance. Photomicrograph, × 100. Figure 7: Female fixed in Bouin five months after nerve severance. Photomicrograph, × 100. Figures 8 and 9: Male fixed in Bouin six months after operation. The contrast between "severed" (Fig. 8) and normal (Fig. 9) corpus allatum is particularly marked. Photomicrograph, × 375.

Gomori technique. It also shows that the corpus cardiacum on the operated side is smaller than on the normal side. This effect of nerve severance on the corpus cardiacum is different from that observed on the corpus allatum (see below) and may be the result of prolonged inactivity as a storage center. The posterior part of the corpora cardiaca of specimens with unilateral operation may contain neurosecretory substance on both sides, but since the organs are continuous at the posterior end the material on the operated side probably is derived from the intact side.

II. *The effect of the severance of the nervi corporis cardiaci on the structure of the corpora allata*

In histological preparations, the corpora allata of normal specimens of Leucophaea are, as a rule, conspicuous by the density of their nuclei which make the stained organs look dark. After severance of the nervi corporis cardiaci the histological appearance of the corpora allata changes markedly. The organs appear larger and lighter in color. This effect is brought about by an increase in cytoplasm relative to the number of nuclei per unit of area. The nuclei are especially loosely arranged in the periphery of these organs. It seems that the number of nuclei per gland remains the same after nerve severance, and that the increase in organ size constitutes an increase in cytoplasm, which may be due merely to uptake of water or may indicate an actual production of cytoplasmic material. The nuclei also differ from normal ones after nerve severance; in the operated organs they tend to be more vesicular and their nucleoli are more distinct.

The difference between a normal corpus allatum and one whose connection with the brain has been interrupted can be particularly well demonstrated in cases of unilateral nerve severance. The illustrations (Figs. 6 to 9) show organs from animals which had been allowed to survive the operation for five days, five months, and six months respectively. The structural change is already evident a short time after nerve severance; it is especially marked in the case with an interval of six months after the operation.

The size difference between these normal and "severed" corpora allata was roughly estimated in the following manner. In 5 micra serial sections of the organs, camera lucida outline drawings of every fourth section were made. In the case of an imperfect section the one preceding or following it was substituted. The areas in square millimeters were determined planimetrically and the values were added up for each organ measured. The figures for three representative cases were as follows:

		Normal	Operated
Case 737	♀ (int. 5 ds.)	5,954.83 sq. mm.	10,335.46 sq. mm.
Case 424	♀ (int. 5 ms.)	6,148.37 sq. mm.	8,148.37 sq. mm.
Case 465	♂ (int. 6 ms.)	1,122.58 sq. mm.	5,729.02 sq. mm.

The differences between the values for the normal and the operated side indicate a substantial increase in the size of those corpora allata whose nervous connection with the brain had been severed.

DISCUSSION

The observation that the distribution of neurosecretory material in the intercerebralis-cardiacum complex of Leucophaea changes in the manner described after the nervi corporis cardiaci are severed, clearly indicates that normally a transport of this material takes place along the axons of the neurosecretory neurons. This conclusion is further supported by the corresponding data in vertebrates as stated in the introduction. In frogs (Hild, 1951), in rats (Stutinsky, 1951) and in dogs (Scharrer and Wittenstein, 1952) the accumulation of neurosecretory material proximal to the site of severance of the pituitary stalk is evident soon (several days) after the operation and disappears after a longer interval. The same is true in Leucophaea. A depletion of this material in the portion distal to the transection, such as that observed in Leucophaea, has so far not been clearly demonstrated in vertebrates, but attention should be called to the fact that the observation of such quantitative differences is more difficult in animals where the organ system in question is unpaired and consequently no comparison can be made with a control organ in the same animal.

As far as other insects are concerned, a comparable transport of neurosecretory material from the brain to the corpus cardiacum must be assumed to take place in a variety of species where, as in Leucophaea, the occurrence of this material in the nervi corporis cardiaci can be demonstrated (Cazal, 1948; Stutinsky, 1952).

The concept of the transport and storage of neurosecretory material in the corpus cardiacum makes understandable the physiological result observed in Leucophaea after the interruption of the nervi corporis cardiaci (B. Scharrer, 1946a). These experiments gave no indication of an acute hormone deficiency and no deficiency should be expected since the operation does not result in the immediate depletion of neurosecretory material in the components of the system. The extent to which the evaluation of the corpus cardiacum as a reservoir for hormones originating in the brain can explain other known physiological observations has been discussed in some detail elsewhere (B. Scharrer, 1952b, 1952c).

The interpretation of the hypothalamo-hypophyseal system of vertebrates (Bargmann and Scharrer, 1951) and of the intercerebralis-cardiacum-allatum system of insects (B. Scharrer, 1951; 1952b; see also Scharrer and Scharrer, 1944) in this new light has led to a re-examination of a comparable situation in the eyestalk of crustaceans. In this group of arthropods the sinus gland has long been considered as the source of a number of hormones, while the functional role of the X organ remained obscure ever since its discovery by Hanström (1931). Recent investigations show that hormones known to be present in the sinus gland, especially the molt-inhibiting hormone, are only stored there; they actually originate in the neurosecretory cells of the X organ from which they are transported to their reservoir via the sinus gland nerve (Passano, 1951a, 1951b; Bliss, 1951; Travis, 1951; Frost, Saloum and Kleinholz, 1951).

Morphological evidence of the presence of neurosecretory material in this nerve has been obtained in a number of brachyurans (Enami, 1951; Bliss, 1951; Passano, 1951a). Nerve section in crustaceans (Passano, 1951a) seems to have an effect on the distribution of the neurosecretory material comparable to that in insects as reported here. The information available now is sufficient proof for the existence of a third neurosecretory system (X organ-sinus gland system) which corresponds in many details to the neurosecretory systems in insects and in vertebrates.

As to the effect of nerve section on the corpora allata, one may inquire into the nature of the structural changes observed in these organs after their connection with the brain has been severed. Do they represent a specific reaction attributable to the absence of nervous or of endocrine (neurosecretory) stimulation and, if this is so, does the histological response to this operation reflect a change in the physiological activity of the corpora allata?

The reaction to the severance of the nervi corporis cardiaci cannot be explained as an unspecific effect, possibly brought about by the surgical procedure, because if this were true unilateral transection would have to affect both corpora allata in a similar way. Whether the interruption of the nerves in itself or the disturbance in the flow of neurosecretory substance along these nerves causes the change, cannot be decided at this time. There is information, however, regarding the physiological activity of "severed" corpora allata. It has been previously reported (B. Scharrer, 1946a) that the separation from the brain does not render the corpora allata of Leucophaea functionally incompetent. Bilateral section of the nervi corporis cardiaci neither prevents egg maturation nor nymphal molting, both being processes governed by corpus allatum hormone. This result is not unexpected in view of the fact that allatum implants can substitute for the intact gland in allatectomized animals (B. Scharrer, 1946b, 1946c). In these cases the isolated corpora allata react like intact glands, *i.e.,* they furnish hormone which reaches the circulation in sufficient amounts to be effective.

A physiological difference between normal and "severed" organs which may concern hormone release rather than hormone production can be observed in last instar nymphs. It is generally assumed that, in the course of insect development, metamorphosis takes place because the corpora allata in the last larval stage no longer release an adequate amount of juvenile hormone. The manner in which this presumed restraining influence on the corpora allata is mediated has not been determined. The effect of the cutting of the nervi corporis cardiaci shows, however, that this regulatory mechanism requires the intactness of the nervous pathway. Last instars of Leucophaea in which the corpora allata are severed from the brain may undergo supernumerary molts and become "nymphoids" instead of adults (B. Scharrer, 1946a), an indication that the severed corpora allata can release a significant amount of juvenile hormone at a stage when they normally would not do so. This result can be interpreted in several ways. Under normal conditions the brain may regulate the release of corpus allatum hormone by means of the nervi corporis cardiaci. In view of the peculiar nature of this pathway the action on the corpora allata can be either nervous or neuro-endocrine in character. Another possibility is that the potency of the severed corpus allatum is increased to such a degree that the juvenile hormone released during the last stage reaches the threshold necessary for an additional nymphal molt, while normally this level is not reached.

In the latter case the histological changes following nerve severance would have to be interpreted as a sign of increased glandular activity rather than of deficiency. This has been done by some authors who observed structural differences similar to the ones described here in Leucophaea, when they compared the corpora allata of specimens in inactive and active phases of their development (Wigglesworth, 1934; Mendes, 1948), of males and females (Pflugfelder, 1938, 1948; Schrader, 1938; Hanström, 1942; Palm, 1948) or of normal and castrate specimens (Pfeiffer, 1940; E. Thomsen, 1942; Day, 1943; see also Vogt, 1942) of certain insect species.

An effect of nerve severance on the structure of the corpus allatum has been reported only in one other instance. Day (1943) observed an increase in the size of the corpus allatum comparable to the one in Leucophaea, when the "recurrent" nerve of the fly Sarcophaga was cut. In the adult flies this nerve contains both nervi corporis cardiaci at the level of the operation. Therefore, Day's experiment constitutes a denervation of the corpus allatum, while the severance of the nervi corporis cardiaci in Leucophaea does not deprive the corpora allata of fibers they may receive from the stomatogastric system. Whether or not this anatomical difference, determining different degrees of denervation, accounts for the differences in the physiological effects observed in Leucophaea on the one hand and in Sarcophaga on the other is not certain. While the stimulation of the ovaries of Leucophaea by the corpora allata continues after section of the nervi corporis cardiaci this is not the case in Sarcophaga where, according to Day, nerve severance has the same effect as extirpation of the corpus cardiacum-allatum. With respect to Leucophaea one may assume that the physiological effect which parallels the morphological change of the corpora allata following nerve severance constitutes an increase rather than a decrease in glandular activity.

SUMMARY

1. The interpretation of the intercerebralis-cardiacum-allatum system of insects as a functionally related group of neuroglandular organs, in which the corpus cardiacum serves as a reservoir for neurosecretory material, is supported by the results of severance of the nervi corporis cardiaci. The insect, *Leucophaea maderae,* in which the components of this organ system are paired is favorable for the study of these relationships since one side can be left intact and compared with the operated side.

2. Following the interruption of the neurosecretory pathway at the level where the nervus corporis cardiaci enters the corpus cardiacum, the distribution of the neurosecretory material changes markedly in comparison with the normal situation. Proximal to the site of nerve section there is an accumulation, and distal to it there is a drastic depletion of neurosecretory material. This result offers conclusive evidence that in the intact system the neurosecretory substance originating in cells of the pars intercerebralis of the brain is transported via their axons (forming the nervi corporis cardiaci) to the corpora cardiaca where it is stored. This interpretation of the morphological situation makes it understandable that no physiological indication of hormone deficiency was observed as a consequence of the nerve transection (B. Scharrer, 1946a).

3. Severance of the nervi corporis cardiaci also results in histological changes in the corpora allata. The organs become larger and seem to contain more cytoplasm than normal glands. Their nuclei are more vesicular and the nucleoli more distinct. These changes do not indicate an organ deficiency, since "severed" corpora allata of Leucophaea are functionally equal to, or perhaps even more competent than, normally innervated glands.

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THE EFFECT OF SALTS AND ORGANIC SOLUTES ON THE MIGRATION TIME OF THE SLIME MOLD DICTYOSTELIUM DISCOIDEUM[1]

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As Raper (1935) showed when he first described the amoeboid slime mold *Dictyostelium discoideum*, this particular species is unique in that it possesses a migration stage in its life cycle. The independent, separate amoebae of the vegetative stage stream together to form cell masses or pseudoplasmodia, and these pseudoplasmodia migrate over the surface of the substratum until the final culmination stage. In culmination there is both a rising into the air and a cell differentiation; the result is a fruiting body in which a delicate stalk made up of large vacuolate amoebae supports an apical mass of encapsulated amoebae or spores.

The duration of this migration period has always been known to be extremely variable, from a matter of a few hours to approximately two days, and Raper (1940) demonstrated that low temperature and high humidity favored prolonged migration to some extent. While these observations have been confirmed we have also found that the concentration of solutes in the medium proves to be a far more important limiting factor, and by varying this environmental condition it has been possible to control and to greatly extend the duration of migration.

METHODS

The organisms were cultivated on standard medium (Bonner, 1947)[2] with *Escherichia coli* at room temperature for two days, at which time the amoebae had usually begun to aggregate. Plugs of the standard agar covered with the aggregating amoebae were cut out with a glass ring 10 mm. in diameter and placed on a circular coverslip of equal diameter, which was then set on the medium to be tested. The coverslip was used so that no diffusion could take place between the plug of standard medium and the test medium. All the test plates were incubated at 17° C. The technique thus far is essentially similar to that used by Bonner *et al.* (1950).

After 24 hours a sufficient number of migrating pseudoplasmodia had moved on to the test medium so that the plug and coverslip could be removed. Each of the migrating pseudoplasmodia that remained was marked by placing a drop of ink on the bottom of the petri dish at 24-hour intervals until fruiting occurred. In cases where the pseudoplasmodia split longitudinally all the parts were followed.

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[1] The preliminary experiments of this study were carried out at Princeton University with the help of a grant from the American Cancer Society.

[2] There is an error in the formula of the culture medium in Bonner (1947) and KH_2PO_4 should be substituted for the incorrect K_2HPO_4.

The test media were prepared by adding the different solute concentrations to 2% Difco Bacto-Agar and each solute was tested separately. All solutes were chemically pure.

RESULTS

First a test was made using the standard medium. As can be seen from the table, in 137 cases the mean migration time was 2.0 days (± a standard deviation of 1.3) and the range extended from 1 to 5 days. This is in keeping with the previous reports in the literature, but if the migrating pseudoplasmodia were allowed to migrate in 2% agar alone, the mean time was extended to 10.3 days (see table) and in one instance the migration lasted 20 days. As a matter of fact the mean value is in this instance of dubious significance because 29% of the pseudoplasmodia disappeared completely, presumably from a combination of loss of cells by straggling in the posterior track and the loss of protoplasm used in the energy of locomotion. The smaller the original size of the pseudoplasmodium, the sooner it would disappear, but there was no evident correlation between duration of migration before fruiting, and the size of the pseudoplasmodium.

As can be seen from the table and from Figure 1, if any solute was added to the 2% agar, the greater the concentration of the solute the shorter the mean duration of migration. This was true for the three organic non-electrolytes tried (dextrose, sucrose, dl-alanine) and the three electrolytes (NaCl, Na_2HPO_4, $CaCl_2$). None of the non-electrolytes was significantly different from any other, but as a group the salts were from 4 to 6 times more effective at a given concentration. Among the salts, NaCl and $CaCl_2$ were very similar in their effectiveness, while Na_2HPO_4 was twice as effective.

If concentrations of solutes higher than those given in the table were used, only a few pseudoplasmodia crawled off the standard medium plug even though they readily left plugs from the same standard plate when put on media of lower concentrations. There was one exception to this: in the case of the 6% dextrose a good number of pseudoplasmodia migrated off the plug, but as soon as they touched the surrounding medium they fruited. This happened in 78 cases without any deviations and represents a condition of minimum migration. It was also noticed on repeated occasions that prolonged migration never occurred over the surface of glass, but that if the pseudoplasmodium reached the side of the petri dish, it fruited shortly thereafter.

DISCUSSION

The cessation of migration and the onset of fruiting is a major step in the differentiation process of *Dictyostelium*. It is of interest therefore to observe that the concentration of the solutes can play a part in the initiation of differentiation. Considering the importance of concentration, the effect would appear to be an osmotic phenomenon, but this would neither account for the more effective nature of the salts, nor the fact that the salts differ among themselves when their effective osmotic pressure is calculated. In the future it is hoped that by testing other substances it will be possible to understand more thoroughly the mechanism of initiation of fruiting and the basis for the variability of effectiveness of different substances.

There is a parallel to the phenomena found here in the work of Smith and Grenan (1949), who have found that the concentration of the medium will affect

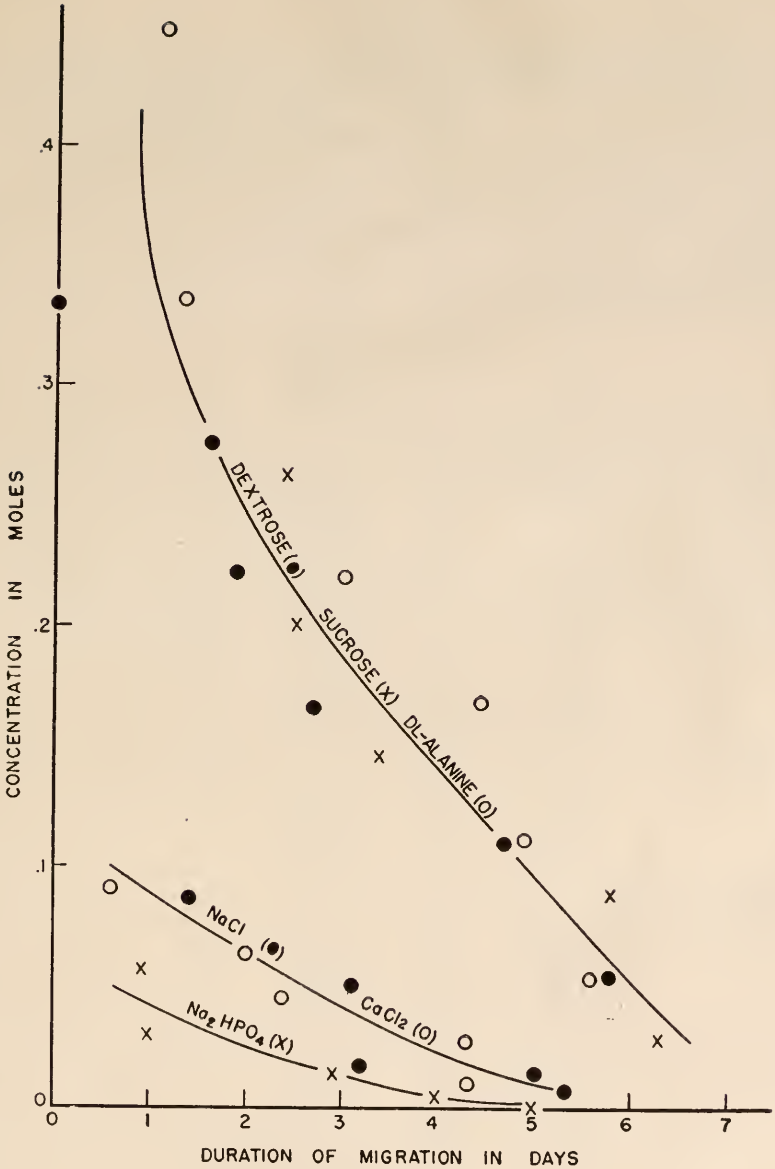

FIGURE 1. A graph showing the relationship between the concentration of solute (moles) in a 2% agar test medium and the duration of migration of the slime mold *Dictyostelium discoideum* in days.

the degree of spreading of the plasmodium in the true Myxomycete, *Physarum polycephalum*. Again with increasing concentrations there is a decrease in spreading, which suggests the possibility that in *Dictyostelium* the solutes affect the motility of the cell mass and therefore indirectly initiate final differentiation.

TABLE I

The effect of solutes on the migration time of Dictyostelium discoideum

% Weight of solute in 2% agar	Conc. in moles	Mean time migration in days	Standard deviation	Migration in days		No. of cases
				Max.	Min.	
Std. medium		2.0	±1.3	5	1	137
2% Agar alone	0	10.3	±5.40	20	1	142
1% dextrose	.056	5.8	±2.71	11	1	79
2%	.11	4.7	±2.33	11	1	53
3%	.17	2.7	±1.30	8	1	154
4%	.22	1.9	±0.92	4	1	56
5%	.28	1.6	±0.54	3	1	53
6%	.33	0	0	0	0	78
1% sucrose	.029	6.3	±3.02	13	1	120
3%	.088	5.8	±2.40	12	1	59
5%	.15	3.4	±2.05	9	1	58
7%	.20	2.5	±2.28	10	0	29
9%	.26	2.4	±1.70	6	0	33
0.5% dl-alanine	.056	5.6	±2.34	11	1	82
1%	.11	4.9	±2.02	10	0	94
1.5%	.17	4.4	±2.07	8	0	64
2%	.22	3.0	±1.74	7	0	59
3%	.34	1.3	±0.99	3	0	43
4%	.45	1.1	±0.80	3	0	17
0.05% NaCl	.0086	5.3	±2.41	11	1	73
0.08%	.014	5.0	±2.04	10	1	76
0.1%	.017	3.2	±1.76	7	1	90
0.3%	.051	3.1	±1.28	6	1	71
0.5%	.086	1.4	±0.75	4	1	74
0.05% $Na_2HPO_4 \cdot 12\ H_2O$	.0014	5.0	±2.64	11	1	109
0.1%	.0028	4.0	±2.24	10	1	49
0.5%	.014	2.9	±1.92	8	1	141
1%	.028	1.0	±0.42	2	0	113
2%	.056	0.9	±0.54	2	0	37
0.1% $CaCl_2$	.0090	4.3	±2.60	11	1	63
0.3%	.027	4.3	±2.01	8	0	63
0.5%	.045	2.4	±1.53	6	0	47
0.7%	.063	2.0	±1.15	4	0	26
1.0%	.090	0.6	±0.06	2	0	24

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SUMMARY

1. In the life history of *Dictyostelium discoideum* there is a period of migration in which the aggregated cell mass wanders about in the form of a sausage prior to the formation of a final fruiting body.

2. The period of migration was known to be variable, and now a limiting factor has been demonstrated. It was possible to show that in the absence of solutes migration will occur for long periods of time, and that the duration of migration decreased approximately exponentially with the concentration of added solute. The effect cannot be accounted for entirely as an osmotic phenomenon because different substances have different degrees of effectiveness, electrolytes being more effective than non-electrolytes.

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THE EFFECT OF PARROT FISH THYROID EXTRACT ON THE RESPIRATORY METABOLISM OF THE WHITE RAT [1, 2]

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The discovery of a discrete, encapsulated thyroid gland in the parrot fish (Matthews, 1948) made possible studies on the teleost thyroid which had hitherto been undertaken only with the greatest difficulty. Extracts of the fish thyroid became obtainable for the first time with relative ease. Such extracts, when injected into white grunts, significantly elevated the oxygen consumption in a certain proportion of the fish so treated (Smith and Matthews, 1948). The thyroids of parrot fish were found to concentrate iodine in a manner reminiscent of similar behavior in the glands of mammals (Matthews and Smith, 1948). There was thus established a certain parallelism between the action of teleost and mammalian thyroids and it was thought worthwhile to investigate the effect of parrot fish thyroid extract upon the respiratory metabolism of the white rat.

METHODS

The extracts used in these studies were prepared from thyroid glands collected in Bermuda and Bimini. With rare exceptions, the glands were taken from *Pseudoscarus guacamaia*. As previously described (Smith and Matthews, 1950), the thyroid glands were removed immediately after the death of the fish and were placed in either acetone or absolute alcohol. Dehydration and defatting continued for at least two days with alternate changes of acetone or absolute alcohol. At the end of this period the glands were dried in partial vacuum and stored, either powdered or whole, in a desiccator until prepared for injection. The final average weight of the glandular material was 17 per cent of the wet weight of the gland when removed from the fish.

Extracts were prepared by dissolving the desired amount of dried glandular tissue in 2 cc. of 2N NaOH and bringing the resultant solution to neutrality with concentrated HCl. This procedure usually precipitated most of the dissolved material, but the resultant suspension was fine enough to be drawn through a 20 gauge needle and injected into the test animal. As a rule approximately 100 mgm. of dried gland were prepared in this manner. The final volume of solution injected amounted to about 2.0 cc. This solution was injected intra-abdominally into adult male white rats, bred from laboratory stock. The rats stood the injection well and showed no sign of distress immediately afterward or at any later time. The animals were kept in individual cages under laboratory conditions during the time necessary to complete the experiment. During this period they were given the usual food and water *ad libitum*.

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[1] With the support of a grant from the American Philosophical Society.

[2] Contribution No. 173, Bermuda Biological Station for Research.

Mammalian thyroid extracts were prepared in the same manner, the necessary amount of commercial desiccated mammalian thyroid (tablet form) being dissolved in NaOH and neutralized with HCl. The synthetic thyroxine used in these experiments was prepared by Roche-Organon, one cc. of the solution containing 2 mgm. of thyroxine. Material for control injections was prepared by adding to the required amount of NaOH sufficient concentrated HCl to bring the pH of the solution to about 7.0.

Oxygen consumption was determined by placing the rat in a desiccator (volume 9500 cc.) through which air was forced under a slight positive pressure sufficient to give a flow of 500 to 670 cc. per minute. The flow was adjusted so that CO_2 concentration at equilibrium usually fell between 0.7 and 1.3 per cent. The animals quickly became accustomed to the apparatus and after briefly exploring their surroundings settled down and usually spent most of the time sleeping throughout the remainder of the test. Samples of air were taken after the rats had been in the chamber for two hours. They were analyzed with a Haldane-Henderson gas analyzer to determine the amount of oxygen which was removed and the amount of carbon dioxide which was added to the air during its passage through the chamber, the rate of flow at the time of sampling having been previously determined. It was then possible to calculate both oxygen consumption and CO_2 production during the test. The respiratory quotient and the oxygen concentration per hour per gram of body weight were then determined. The validity of the method is predicated, of course, on the assumption that no sudden change in oxygen consumption occurred during the test period, particularly within 10 minutes before collection of the sample.

The tests were run in the morning, after the rats had been left overnight in their cages with food and water. They were not fasted, therefore, prior to the test, since it is well known that rats under laboratory conditions usually feed at night and spend the daylight hours in sleep. Since repeated tests were desired on successive days extending over a period of a week or more, depriving the rats of food for this period would have produced such abnormal behavior as to invalidate the results. The relative constancy of the oxygen consumption of each rat over a period of several days prior to any experimental procedure indicated that the metabolic conditions prevailing during the tests were reasonably similar.

RESULTS

The purpose of the experiments was to determine the effect of parrot fish thyroid extract on the oxygen consumption, respiratory quotient and weight of adult male white rats and to make a comparison with the effects produced by synthetic thyroxine and desiccated mammalian thyroid powder.

Table I gives the results of 86 determinations of oxygen consumption and R.Q. in 22 non-treated rats. Their weights ranged from 250 to 500 grams (average 300 grams). Average oxygen consumption was 0.98 cc./gm./hr. and the average R.Q. was 0.84. As may be seen from an inspection of Table II, the values for the various test series before injection of thyroid preparations were in every instance close to these figures.

In Table II are shown the results of injecting various substances, known or suspected to contain active thyroid hormone, as compared to the effect of injecting

TABLE I

Oxygen consumption and respiratory quotient in non-treated white rats

Number of determinations	86
O_2 consumption (cc./gm./hr.) Mean ± SE_M	0.98 ± 0.0129
Respiratory quotient Mean ± SE_M	0.84 ± 0.0070

TABLE II

Oxygen consumption and respiratory quotient in treated rats

Rat no.	Material injected	Before injection		Days after injection											
				1		2		3		4		7		10	
		R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.	R. Q.	O_2 cons. cc./hr./gm.
8	Neut.	0.85	0.93	0.85	0.97	0.84	0.97								
4	NaOH	0.84	0.91	0.82	0.97	0.85	1.02								
1	2 cc.	0.82	1.08	0.77	0.93	0.81	1.03	0.84	0.93	0.85	0.80	0.74	0.92		
2A		0.83	1.12			0.76	0.99	0.79	0.91	0.79	0.95	0.84	0.89		
4A		0.78	1.12	0.81	1.01	0.79	1.08			0.81	1.12	0.84	1.01		
6A		0.84	0.98	0.76	1.01	0.81	0.89	0.80	0.91	0.81	0.95	0.79	0.96		
Ave.		0.83	1.02	0.80	0.98	0.81	1.00	0.81	0.92	0.82	0.96	0.80	0.95		
2	Thyroxine	0.76	1.02	0.79	1.34	0.69	1.44	0.72	1.16					0.93	0.99
7	1 mgm.	0.80	1.14	0.74	1.28	0.68	1.36	0.75	1.34					0.86	0.93
11	1 mgm.	0.85	0.91	0.79	1.22	0.69	1.39	0.74	1.15					0.81	0.90
0	0.2 mgm.	0.78	0.95	0.76	1.21	0.74	1.24	0.80	1.00						
13	0.2 mgm.	0.85	0.95	0.73	1.18	0.73	1.06	0.76	1.00						
Ave.		0.81	0.99	0.76	1.25	0.71	1.30	0.75	1.13					0.87	0.94
7A	Mammalian	0.83	0.90	0.69	1.13	0.74	1.08	0.72	0.97			0.94	1.03	0.95	0.88
8A	Thyroid	0.84	1.06	0.80	1.31	0.82	1.25	0.75	1.36	0.78	1.28	0.76	1.45		
	130 mgm.														
Ave.		0.84	0.98	0.75	1.22	0.78	1.16	0.74	1.17			0.85	1.24		
6	Fish	0.90	0.85	0.85	1.18	0.82	1.11	0.84	1.19			0.80	0.97	0.78	1.04
8	thyroid	0.85	0.93	0.80	0.99	0.87	0.92*								
	96 mgm.					0.78	1.23	0.73	1.19						
5	Av. dose	0.91	0.92			0.73	1.06	0.77	1.08						
Z		0.86	0.90	0.71	0.85	0.69	1.02	0.75	1.17	0.78	1.25	0.81	1.25		
K		0.93	1.01	0.77	1.16	0.74	1.38	0.78	1.17	0.75	1.20	0.84	1.10†		
3A		0.87	0.92	0.77	1.13	0.81	1.09	0.84	0.98	0.90	1.05	0.87	0.91		
5A		0.84	0.93	0.79	1.13	0.73	1.15	0.70	1.17	0.79	0.90	0.86	0.85		
Ave.		0.88	0.98	0.78	1.07	0.77	1.12	0.77	1.14	0.81	1.10	0.84	1.02		

* Re-injected with larger dose.

† Died on 8th day.

neutralized NaOH. These experiments are divided into four series, each of which is discussed separately below.

Neutralized NaOH solution. As a control on the handling and injection of the rats, neutralized NaOH solution was injected, in the same amount and manner as were the thyroid preparations. Six rats were so treated and in no instance was there any significant change in R.Q. up to 7 days after the injection. A slight fall in oxygen consumption during the experiment was probably attributable to adaptation.

Thyroxine. Synthetic thyroxine (Roche-Organon) was injected into 5 rats at two dose levels, 1.0 mgm. and 0.2 mgm. There appeared to be no appreciable difference between the effects of these two doses. Maximum effects on oxygen consumption and R.Q. were seen within two days after the injection.

Mammalian thyroid extracts. Commercial desiccated mammalian thyroid was extracted and injected in the manner described above. In two experiments 130 mgm. were given each time. There was a marked elevation of oxygen consumption, and a fall in R.Q. on the first day after the injection which continued until beyond the fourth day.

Fish thyroid extract. Extracts of parrot fish thyroid glands prepared in the manner already described were injected intra-abdominally into seven rats. The amount of dried gland extracted for this purpose varied between 69 and 110 mgm. In rat No. 8, 69 mgm. failed to produce an elevation in oxygen consumption or a fall in R.Q. by the second day after injection. Possibly this dosage was too small. On the second day after the original injection, therefore, the same animal was reinjected with 98 mgm. of dried extracted gland. Two days later it showed a marked rise in oxygen consumption and a fall in R.Q. In all other instances an elevation in oxygen consumption and a fall in R.Q. were observable within the first or second day after injection. In most cases oxygen consumption was still elevated on the seventh day, although the R.Q. usually had returned to the pre-injection level by that time.

Figure 1 shows graphically the average percentage changes in oxygen consumption and R.Q. All thyroid preparations produced increases in oxygen consumption and decreases in R.Q., while those rats injected with neutralized NaOH solution showed no percentage change in R.Q. and a perceptible decrease in oxygen consumption. This decrease is undoubtedly due to the adaptation of the animal to the respiratory chamber. As might be expected, the effect of synthetic thyroxine on oxygen consumption is more marked and of shorter duration than are the effects of extracts of fish and mammalian thyroid. Fish and mammalian thyroid injections increased oxygen consumption about 25 per cent at the maximum and the effects persisted for at least 10 days. There is a suggestion that fish thyroid has a somewhat more prolonged action than has mammalian thyroid. This also seems to be true of the effect on R.Q., which was still depressed 10 days after fish thyroid injection, but after mammalian thyroid injection it had returned to the pre-injection level within 7 days. The quantitative differences in the effects of synthetic thyroxine and extracted mammalian and fish thyroid are undoubtedly due to variations in the rate of absorption of the injected material.

No attempt has been made to determine the relative strengths of fish and mammalian thyroid. Since the amounts of the two extracts producing similar effects were of the same order of magnitude, they are probably of the same approximate strength.

Figure 2 shows the effect of fish thyroid extract upon the weight of injected rats as compared to the effect of neutralized NaOH. In the four cases graphically por-

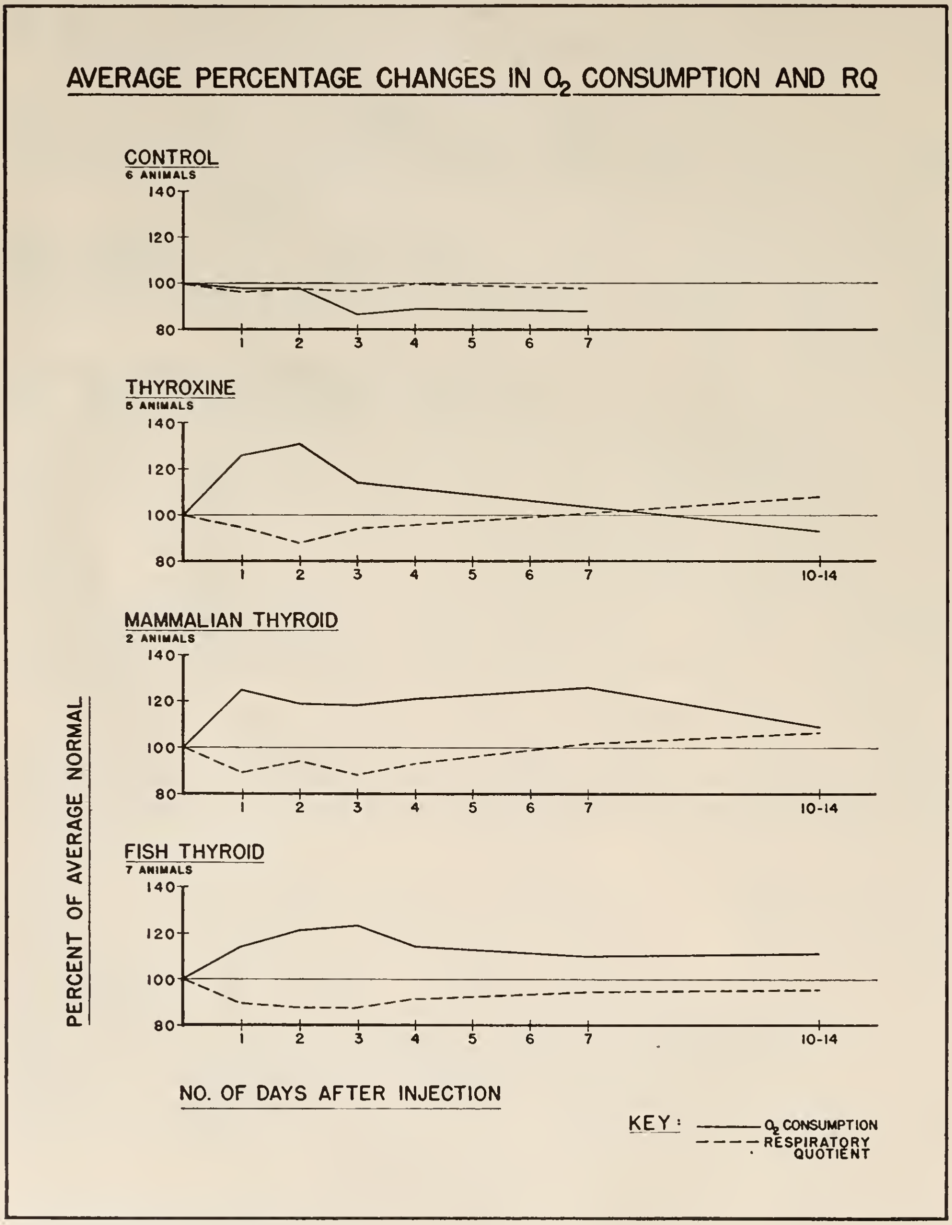
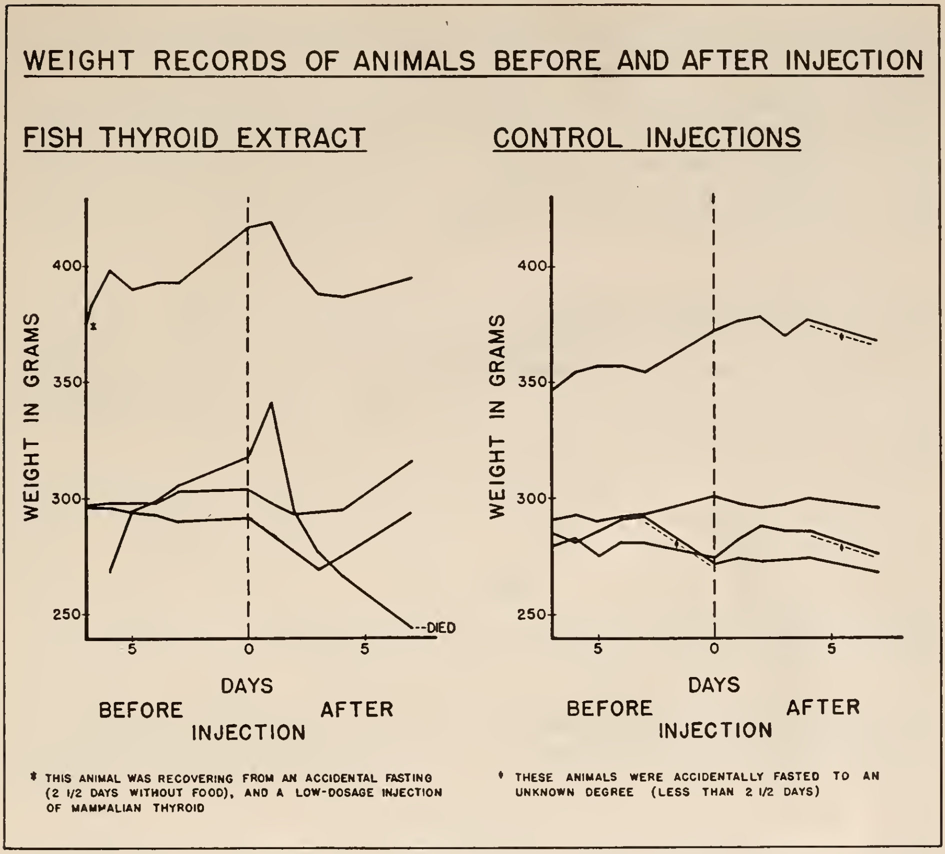


FIGURE 1. Showing the effect of thyroid extracts of fish and mammalian origin, as well as effect of thyroxine, on the oxygen consumption and respiratory quotient of white male rats.



* THIS ANIMAL WAS RECOVERING FROM AN ACCIDENTAL FASTING (2 1/2 DAYS WITHOUT FOOD), AND A LOW-DOSAGE INJECTION OF MAMMALIAN THYROID

♦ THESE ANIMALS WERE ACCIDENTALLY FASTED TO AN UNKNOWN DEGREE (LESS THAN 2 1/2 DAYS)

FIGURE 2. Showing effect of parrot fish thyroid extracts on the weight of white male rats.

trayed, fish thyroid extract invariably produced a marked fall in weight on the first or third day after injection, the fall persisting for at least 4 days. In most instances the weight was not recovered until at least one week after the injection. In those rats which were injected with NaOH solution, the weight of the animal following injection was, beyond the random variations, no different from the pre-injection value. While the effects of synthetic thyroxine and mammalian thyroid extract are not shown in this figure, their effects on weight are the same as that of the fish thyroid extract.

DISCUSSION

Data on the effect of thyroid extracts derived from the glands of lower vertebrates are virtually non-existent. The work done, however, is adequately considered in three excellent reviews on the comparative physiology of the thyroid which have appeared recently (Fleischmann, 1947; Goldsmith, 1949; and Lynn and Wachowski, 1951). These papers cover the subject so thoroughly that there is little point in reviewing the literature here except to summarize it briefly. Fleischmann (1947) notes only the effect of transplanted amphibian glands on amphibian metamorphosis. There seems to be nothing else in the literature on the effect of amphibian extracts and nothing on the effect of reptilian extracts. Concerning the effects of fish thyroid extracts on fish, the only experiments reported are those of Smith and Matthews (1948) on the effect of parrot fish thyroid extracts on the oxygen consumption of white grunts. As to the chemistry of the thyroid hormones of lower vertebrates, we have only the data of Wolff and Chaikoff (1947) on shark and turtle thyroids, which give the thyroxine content in per cent of total iodine in sharks as 27.9 and in the turtle as 31.8, figures which are similar to those found in warm-blooded forms.

Sembrat (1927) reported acceleration of metamorphosis of tadpoles when implanted with bits of thyroid taken from either the dog-fish or the carp. Desiccated parrot fish thyroids (mixed with flour to form a paste) when fed to tadpoles will also produce premature metamorphosis according to Matthews and Ash (1951). Thyroid extracts prepared from glands taken from fish treated with propylthiouracil had no such effect. Thus, there is no doubt that the factor from the fish thyroid producing metamorphosis in amphibians is a true thyroid hormone.

While it is useless to speculate on the function of the thyroid in fishes because of the paucity of experimental data, it is not necessary to assume that it must serve a respiratory function associated with the regulation of body temperature. All attempts to increase the oxygen consumption of fish with mammalian thyroid extracts have failed (Drexler and Issekutz, 1935; Etkin, Root and Mofshin, 1940; Hasler and Meyer, 1942; Smith and Everett, 1943; and Matthews and Smith, 1947). However, extracts of parrot fish thyroid have been shown to elevate oxygen consumption in white grunts (Smith and Matthews, 1948). Since the increase was not consistently found in all injected grunts, there is some doubt as to the physiological significance of the elevation observed. It is not impossible that the respiratory stimulation is due to some sort of toxic reaction of the injected material. Nevertheless, the implication remains that teleost thyroid extract can elevate oxygen consumption and mammalian cannot. Matthews and Smith (1947) were unable to observe any change in oxygen consumption in Fundulus when injected with thiourea over a period of 5 to 6 days. It is possible, of course, that longer treatment with thiourea might have produced a different result. Further studies are necessary, including observations on the effect of fish thyroid extracts and thiourea on the R.Q. of fishes, before a final conclusion can be drawn.

There seems little doubt that the increase in respiratory metabolism observed in white rats after injection of thyroid extracts of the parrot fish thyroid is due to increased cellular oxidation. The exact parallelism between the effect of thyroid extract of mammalian origin as compared to that of fish origin leaves little room to question that the two are much alike in their physiological effects in the mammal. This view is strengthened by the fact that synthetic thyroxine behaves in the same way. The rise in oxygen consumption, fall in R.Q. and decrease in weight all follow the same pattern, regardless of the source of the thyroid hormone. This finding, coupled with the observations that (1) both desiccated fish thyroids and extracts of these glands will produce premature metamorphosis in amphibians, (2) the fish thyroid will concentrate iodine, and (3) chemically the thyroids of those lower vertebrates that have been studied (the shark and the turtle) are very similar to the glands of higher vertebrates, makes it likely that the thyroid hormone of all vertebrates is much the same. It is, therefore, obvious that the teleost during the course of evolution has not sufficiently changed the nature of its thyroid hormone so that it may be distinguished from that of other vertebrates by ordinary physiological tests on amphibians and mammals. Whether the hormone still serves the same function in teleosts that it did in ancestral forms is at present unknown, just as it is not known whether the function of the hormone in mammals is a recent adaptation. The adaptation, if true, would appear to be on the part of the mammalian tissue to the hormone, rather than the other way around.

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SUMMARY

1. The effect of injecting extracts of parrot fish thyroid into adult, male white rats was to increase oxygen consumption, decrease respiratory quotient and decrease weight.

2. Injections of synthetic thyroxine or extracts of desiccated mammalian thyroid produced similar effects in respect to time of onset, intensity and duration of the responses.

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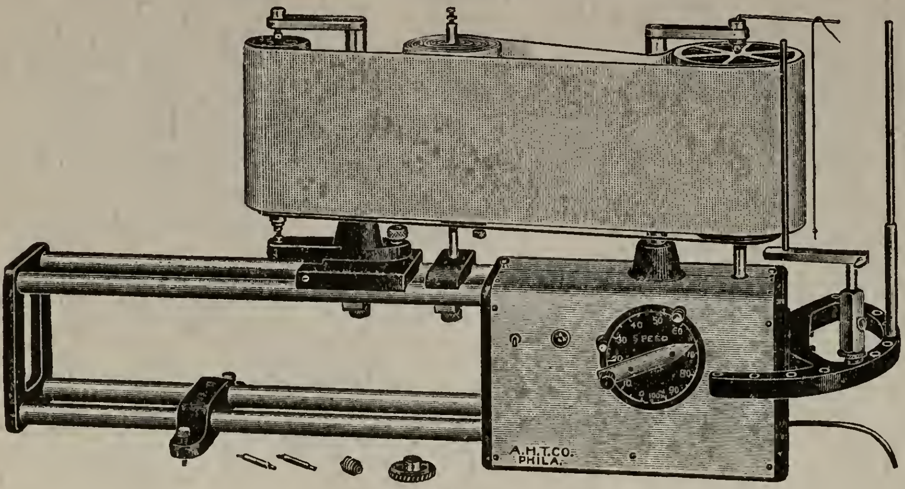

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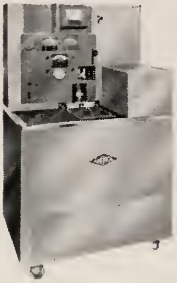

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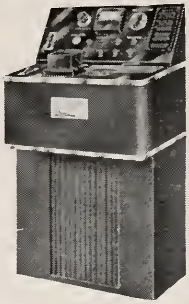

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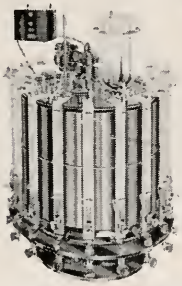

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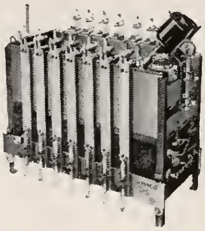

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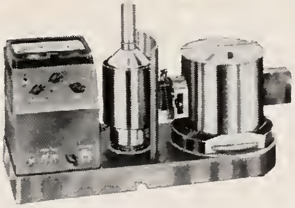

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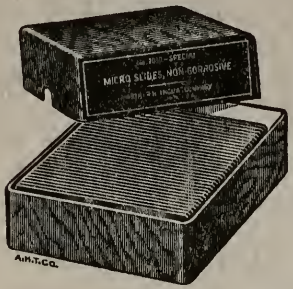

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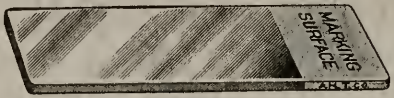

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